Glycine nitrosation and signature mutations in the p53 tumour suppressor gene: a molecular link between diet and cancers of the gastro-intestinal tract.

Thesis

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Glycine Nitrosation and Signature Mutations in the p53 Tumour Suppressor Gene – A Molecular Link between Diet and Cancers of the Gastro-Intestinal Tract

A Thesis submitted for the degree of
Doctor of Philosophy
To
The Open University

By

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Abstract

GLYCINE NITROSATION AND SIGNATURE MUTATIONS IN THE p53 TUMOUR SUPPRESSOR GENE – A MOLECULAR LINK BETWEEN DIET AND CANCERS OF THE GASTO-INTESTINAL TRACT

Genotoxic agents derived from diet may contribute to the total human burden of potentially carcinogenic DNA damage. For example, glycine is a simple primary amino acid and a commonly found dietary constituent in the red meat. The nitrosation of glycine would probably constitute a major source of alkylating agents, as glycine is one of the most abundant amino acids found in nature. It has been shown that O6-carboxymethyl-2'-deoxyguanosine, the major O6-guanine adduct of nitrosated glycine derivatives, is not repaired by O6-alkylguanine-DNA-alkyltransferase so could accumulate in the DNA of GI tract tissues as a promutagenic lesion. In DNA extracted from human gastric biopsies and white blood cells, using a sensitive Immunoblot assay, O6-CMdG adduct has been detected suggesting that the formation of carboxymethylating agents, possibly via nitrosation of glycine, does occur in man. Therefore genotoxic properties of the O6-carboxymethyl-2'-deoxyguanosine adduct were studied.

O6-carboxymethyl-2'-deoxyguanosine adduct has been successfully synthesised and incorporated site specifically in to oligonucleotides. Nucleoside composition analysis of site specifically modified sequences confirmed the presence of O6-carboxymethylguanine adduct. The melting curves studies showed qualitative differences between base pair stabilities formed by O6-CMdG adduct with respect to O6-MedG adduct and normal 2'-dG. O6-CMdG:T base pair was stable than O6-CMdG:C base pair suggesting that O6-CMdG adduct like O6-MedG might preferentially base pair with thymidine. However, quantitative studies on the melting temperatures of O6-CMdG adduct were not fully conclusive. Clearly further effort and experimentation is needed to substantiate the qualitative data.

The mutagenic potential of O6-carboxymethyl-2'-guanosine adduct was studied using Amplification Refractory Mutation System (ARMS) PCR technique. A shuttle vector was constructed by incorporating a single O6-CMdG adduct site specifically in to p53 cDNA of plasmid, pLS76. The mutagenesis studies have indicated that O6-carboxymethyl-2'-deoxyguanosine adduct can induce both GC→AT transition mutations and GC→TA transversion mutations. Further studies are needed to focus on mutation specificity and frequency of O6-CMdG adduct. The mutagenesis studies on potassium diazoacetate, model compound of nitrosoglycine derivatives did not induce any functional mutations in the p53 gene of embryonic fibroblast cells of Human p53 knock in (HUPKI) mouse. However, this is mainly due to problems associated with KDA stability rather than the toxicity of the compound.

The results presented in this thesis highlight some key aspects of methodology as well as the significance of mutagenecity of O6-CMdG adduct with respect to diet-related cancer risk.
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<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetyl</td>
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<td>ABH</td>
<td>Alkylated DNA Repair protein alkB Homologue</td>
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<tr>
<td>ADE2</td>
<td>Phosphoribosylaminoimidazole carboxylase</td>
</tr>
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<td>AGT</td>
<td>Alkylguanine transferase</td>
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<tr>
<td>AP</td>
<td>Apurinic</td>
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<td>APNG</td>
<td>n-(N-Acetyl-L-Prolyl)-N-Nitrosoglycine</td>
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<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
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<td>ARMS</td>
<td>Amplification Refractory Mutation System</td>
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<tr>
<td>AS</td>
<td>Azaserine</td>
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<tr>
<td>Atase</td>
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<td>Azathioprine</td>
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<tr>
<td>Bcl2</td>
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<td>Bax2</td>
<td>Bcl2 associated X-protein</td>
</tr>
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<td>BPDE</td>
<td>Benzo(a)pyrene diol epoxide</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CCNU</td>
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<td>Carboxymethylguanine</td>
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<td>Calf Thymus DNA</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DMAP</td>
<td>Dimethylaminopyridine</td>
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<td>DMF</td>
<td>N,N-Dimethylformamide</td>
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<td>Death Receptor 5</td>
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<td>EBI</td>
<td>European Bionformatics Institute</td>
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<td>Ec</td>
<td>Esophageal Cancer</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia Coli</td>
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<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid (disodium salt)</td>
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<td>ELISA</td>
<td>Enzyme linked immuno absorbent assay</td>
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<tr>
<td>ENU</td>
<td>N-ethyl-N'-nitrosourea</td>
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<td>EPIC</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>EtdG</td>
<td>O6-ethyl-2'-deoxyguanosine</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
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<td>G</td>
<td>Guanine</td>
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<td>GRE</td>
<td>Glucocorticoid responsive element</td>
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<td>HIPK2</td>
<td>Homeodomain-interacting protein kinase-2</td>
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<td>HCC</td>
<td>Hepatocellular carcinomas</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
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<tr>
<td>HNO₂</td>
<td>Nitrous acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
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<tr>
<td>HUP</td>
<td>Hupki embryonic fibroblast cell lines</td>
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<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>iPr</td>
<td>isopropyl</td>
</tr>
<tr>
<td>ISB</td>
<td>Immunoslot blot</td>
</tr>
<tr>
<td>KDA</td>
<td>Potassium diazooacetate</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium dihydrogen phosphate</td>
</tr>
<tr>
<td>LFS</td>
<td>Li-fraumeni syndrome</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MAA</td>
<td>Mesyloxy acetic acid</td>
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<td>MDA</td>
<td>Malondialdehyde</td>
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<td>Murine double minute chromosome clone number 2</td>
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<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>MedG</td>
<td>Methyl-2'-deoxyguanosine</td>
</tr>
<tr>
<td>MeG</td>
<td>Methylguanine</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl methanesulphonate</td>
</tr>
<tr>
<td>MNNG</td>
<td>N-methyl-N'-nitro-N-nitrosoguanidine</td>
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<tr>
<td>MNP</td>
<td>Mononitrosopipperazine</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>mt</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>NC</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>NDDELA</td>
<td>N-nitrosodiethanolamine</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localisation Signals</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-nitrosodimethylamine</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NMSC</td>
<td>non-melanoma skin cancer</td>
</tr>
<tr>
<td>NMOR</td>
<td>N-nitrosomorpholine</td>
</tr>
<tr>
<td>NMU</td>
<td>N-methyl-N-nitrosourea</td>
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<tr>
<td>NNK</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone</td>
</tr>
<tr>
<td>NNN</td>
<td>N-nitrosonornicotine</td>
</tr>
<tr>
<td>NPRO</td>
<td>N-nitrosoproline</td>
</tr>
<tr>
<td>NPYR</td>
<td>N-nitrosopyrrolidine</td>
</tr>
<tr>
<td>NQO1</td>
<td>quinone oxidoreductase 1</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOG</td>
<td>N-nitrosoglycocholic acid</td>
</tr>
</tbody>
</table>
Abbreviations

NOC  N- nitroso compounds
NOS  Nitric oxide synthase
NOXA/hAPR  (ATL-derived PMA-responsive peptide)
OD  Optical density
Pac  Phenylacetyl
PAGE  Polyacrylamide gel electrophoresis
PBS  Phosphate buffered Saline
PAH  Polyaromatic Hydrocarbons
PCR  Polymerase Chain Reaction
PI  Propidium Iodide
PIDD  p53 protein induced, with death domain
PERP  p53 apoptosis Effector related to PMP22
PUMA  p53 up regulated modulator of apoptosis
RNA  Ribonucleic Acid
RNase A  Ribonuclease A
RNase T  Ribonuclease T
RT  Room Temperature
SDS  Sodiumdodecyl sulphate
SH-3  Src Homology
SNP  Single nucleotide polymorphism analysis
Sn1 (or) 2  Nucleophilic substitution
SSC  Saline Sodium Citrate Buffer
T  Thymidine
TAE  Tris-Acetate-EDTA Buffer
TBE  Tris-Borate-EDTA Buffer
TCR  Transcription Coupled Repair
TE  Tris-EDTA
TEA  Triethylamine
TEAA  Triethylammonium acetate
TEMED  N,N,N',N'-Tetramethylethylenediamine
TFA  Trifluoroacetic acid
THF  Tetrahydrofuran
TLC  Thin Layer Chromatography
UV  Ultraviolet
WHV  Woodchucks hepatitis virus
XP  Xeroderma Pigmentosum
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STATEMENT OF DECLARATION

I declare that the work included in this thesis was carried out by me, the author, during the period of October 2001 to September 2005 at the Department of Chemistry, The Open University (OU), Milton Keynes, UK under the supervision of Prof. David E.G. Shuker and Dr. Yao-Zhang Xu. The results presented in this thesis are from my own investigations.

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Chapter 1: Introduction

1.0 Introduction

DNA damage is an important cause of genetic disease. If the damage involves the genes that control cell growth, it can lead to the development of cancer. Of course, DNA damage may also result in cell death, which can have serious consequences for the organism of which the cell is a part, for example, loss of irreplaceable neurons in the brain. Accumulation of damaged DNA has also been considered to contribute to some of the features of aging. It is not surprising that a complex set of cellular surveillance and repair mechanisms have evolved to reverse the potentially deleterious damage that would otherwise destroy the precious blueprint for life. An increasing number of human hereditary diseases that are characterised by severe developmental problems and/or a predisposition to cancer have been found to be linked to deficiencies in DNA repair. The chemical events that led to DNA damage include hydrolysis, oxidation and electrophilic attack. These reactions are triggered by exposure of cells to exogenous chemicals (e.g. environmental agents, food constituents etc), or they can result from endogenous metabolic processes. Both exogenous carcinogens and endogenous biological processes are known to cause mutations. Classes of DNA damage include deletion, insertion, and base substitution (either transition or transversion). Transitions are base pair substitution mutations in which a pyrimidine replaces a pyrimidine (C→T) or purine replaces a purine (A→G). Transversions are base pair substitution mutations in which a purine replaces pyrimidine (T→G) or a pyrimidine replaces a purine. (A→C). Important sources, which generate point mutations spontaneously in human cells, include deamination of cytosine and 5-methylcytosine, DNA polymerase infidelity, depurination and oxidative damage by free radicals generated from biological processes. Apart from these processes, any abnormalities in the epigenetic mechanisms may cause
deregulation of gene expression. For example, if methylation of CpG islands is disturbed due to deamination or depurination this can lead to GC→AT transitions. A well studied mechanism by which chemical carcinogens and their ultimate carcinogenic metabolites cause mutations is by forming covalent adducts with the nucleotides in the DNA, increasing the possibilities of the errors during DNA replication. Very wide levels of $O^6$-methyl-2'-deoxyguanosine ($O^6$-MedG) adduct have been detected in human DNA in several studies of various populations (Krytopoulos, 1998; Povey, 2000). This might be due to consequence of exposure to alkylation agents that are present in smoke or the diet or to unknown exogenous agents, endogenous processes also contribute to DNA alkylation (Marnett and Burcham, 1993; Hecht, 1999). Despite the indications that a single DNA adduct such as $O^6$-MedG can be responsible for a wide variety of biological effects, the precise molecular mechanisms of recombination and toxicity remained to be defined, whilst that of point mutations is well established. The miscoding properties of $O^6$-MedG had already been established in \textit{in vitro} and \textit{in vivo} polymerase fidelity studies (Loechler et al., 1984; Saffhill et al., 1985). Such mutations occurring in tumour suppressor genes can provide a selective advantage for clonal expansion of proneoplastic and neoplastic cells. Several lines of evidence support the relationship between carcinogenesis and alkylation damage-mediated G→A transition mutations. One is the spectrum of tumour-associated mutations that are found in genes that are crucial for malignant transformation. These include the H-\textit{ras} oncogene in which transition mutations have been reported in codons 12, 13 and 61 (Barch et al., 1991; Rumsby et al., 1991) and the TP53 tumour suppressor gene, where transitions are found in a number of locations (Ohgaki et al., 1992). These mutations are dependent on exposure to alkylation agents (Sukumar et al., 1983).
Figure 1.1: Biomonitoring of exposure to genotoxic compounds. The formation and prevalence of DNA adducts is dependent on a multitude of events such as absorption of the genotoxic compound, its distribution to different tissues, and if necessary its metabolic activation to reactive intermediates. In general formation of DNA adducts reflect on individual susceptibility to genotoxic agents as well as differences in DNA repair (Courtesy of Gottschalk et al., 2006)

Exposure assessment can be carried out at different stages in the process that can lead to the development of tumour as illustrated in Figure 1.1. In the search for a common theme underlying the broad range of cancers, p53 has enjoyed a great deal of attention as the most frequently mutated gene in many types of tumours.
1.1 Insights into p53 Tumour Suppressor Gene

1.1.1 p53, A Tumour Suppressor Gene

P53 is a nuclear protein that regulates gene expression. It is located on chromosome 17p13.1. It lies at the heart of stress pathways leading to human carcinogenesis. Any alteration in p53 gene function provides a selective advantage of clonal expansion of proneoplastic and neoplastic cells. It plays a pivotal role in protection against the development of cancer. It is thought to prevent accumulation of genomic alterations by hindering cell proliferation under genomic stress. The two principal functions of p53 are induction of cell cycle arrest and the activation of apoptotic cell death. Keeping p53 function under control is vital for normal cells (Kubbutat and Vousden, 1998).

1.1.2 Structure of p53

The p53 gene consists of 11 exons, of which exons from 2-11 code for a protein of 393 amino acids. The p53 protein can be divided into three domains; they are as follows [Figure 1.2]

1. N-terminal transactivation domain
2. Core domain- sequence specific DNA binding activity
3. C-terminal oligomerisation domain.

More recently, a proline rich domain has been identified between the transactivation domain and sequence specific DNA binding domain. This region is important for some p53 activities that require interaction with SH-3 domain containing proteins. The amino acid sequence of p53 contains five regions (1-V) that are highly conserved among different species (Kubbutat and Vousden, 1998).
Figure 1.2: Primary structure of p53 tumour suppressor protein: The amino acid sequence of p53 protein consists of five regions (I-V) that are highly conserved among different species, indicating that it has an important role in the function of regulation of the protein. Several residues (marked by numbers) within these conserved boxes are often mutated in humans. These mutations destroy the sequence-specific DNA binding activity of p53, suggesting that this function is critical for suppression. P53 is a phospho protein and several in vivo phosphorylation sites have been mapped with red boxes. NLS indicates the position of the nuclear localisation signals (Kubbutat and Vousden, 1998).

1.1.3 Functions of p53

Recent studies investigating the mechanisms underlying the biological activity of the p53 indicate that this protein is involved in regulation of gene expression, DNA synthesis and repair, genomic plasticity, and programmed cell death. It regulates gene expression by acting as a transcription factor and mediates many of its downstream effects by activation or repression of target genes. Numerous p53 target genes that have been implicated to act, as a down stream effectors are cyclin dependent kinase inhibitor, p21\(^{\text{Waf1/Cip1}}\) which induces cell cycle arrest. It activates ribonucleotide
reductase, named p53R2 is associated with DNA repair. It also activates expression of many genes that mediate mitochondrial and death receptor induced pathways [Figure 1.3] (Almog and Rotter, 1997; Ryan et al., 2001).

![Figure 1.3: A model depicting some of the mechanisms that may regulate subcellular localisation, stability and transcriptional activity of p53 suppressor protein (Ryan et al., 2001).]

It induces apoptotic proteins localised to mitochondria such as Bax, NOXA, PUMA and p53AIP1 that trigger cytochrome c release and activation of Apaf-1/caspase-9 apoptosome. The expression of many death receptors such as Fas and Killer DR5, and the death domain containing protein, PIDD is under the control of p53. It also induces expression of PERP, a potential mediator of apoptosis that belongs to PMP-22/gas3 family of proteins that are localised to the endoplasmic reticulum (ER) and Golgi. Many other studies have implicated that it also has a role in embryogenesis, gene
targeting, spermatogenesis and angiogenesis. Its transactivation activity is required for
cell differentiation (Ryan et al., 2001).

1.1.4 Regulation of p53 Stability

The key regulator of p53 is the MDM2 protein, which can inhibit both p53
transcriptional activity and targeting p53 for ubiquitination. MDM2 can function as
E3 ligase, the final component of the enzyme cascade that results in the conjugation
of ubiquitin to proteins, targeting them for degradation by proteosome. MDM2
ubiquitinates both p53 and itself, contributing to the rapid turnover of both proteins.
As MDM2 is a transcriptional target of p53, an auto regulatory feedback loop is
generated in which increased p53 activity leads to increased expression of its own
negative regulator. P53 function depends on nuclear localization, and both nuclear
import, nuclear export of p53 is dependent on its interaction with the microtubule
network, and dynein, a molecular motor indicating that p53 is actively transported
towards the nucleus, where nuclear localisation signals within the carboxy terminus of
p53 allow efficient nuclear import. p53 also contains efficient nuclear export signal
within its carboxy terminus [Figure 1.3] (Ryan et al., 2001).

1.1.5 Post-translational modification of P53/MDM2 – Regulating, Stabilisation
and Activation of p53 protein

It is crucial to stabilise p53 for a cell experiencing genomic stress in order to maintain
homeostasis. The importance of this process is underscored by a variety of process
with which cell disrupts MDM2 in response to stress. These mechanisms include
post-translational modifications of both p53 and MDM2, subcellular redistribution,
inhibition of MDM2 activity and direct repression of MdM2 transcription. Post-
translational modifications of p53 play an important role in both stabilisation and activation of the p53. Phosphorylation of Ser20 by checkpoint kinases, Chk1 and Chk2 in response to ionising radiation is important in abrogating p53-Mdm2 interaction (Chehab et al., 2000; Hirao et al., 2000), (Sheih et al., 2000). Phosphorylation of Thr81 by Jun amino-terminal kinase can lead to p53 stabilisation (Buschmann et al., 2001). Acetylation of p53 may also contribute to p53 stabilisation. The inhibition of cellular deacetylases leads to longer half-life of endogenous p53 (Ito et al, 2001). Mdm2 can inhibit p53 acetylation mediated either by CREB binding protein (CBP) or by highly related p300 protein (Kobet et al., 2000). P53 is ubiquitinated and acetylated on similar sites at the carboxy terminus suggesting that these modifications compete for the same residues. This suggests a possible link between acetylation and stabilisation (Rodriguez et al., 2000). MDM2 can be modified post-translationally to prevent p53-Mdm2 interaction. It becomes hypophosphorylated in conserved region 11 after DNA damage as well as in response to ionising radiation resulting in p53 stabilisation (Blattner et al., 2002).

Ubiquitination represents an essential activity of MdM2 for regulating the amount of p53 protein availability. It acts as an E3 ligase for p53 by linking E2-conjugated ubiquitin molecule to it via an isopeptide bond in the nucleus (Zhang and Yiong, 2001). This is shuttled in to cytoplasm either directly or indirectly by MDM2 and then recognised by the 26S proteosome for further degradation (Gottifredi and Prives, 2001; Michael and Voren, 2002; Vousden and Woude, 2000). Suppressor gene 101 (TSG101) and c-Abl both have a negative effect on p53 ubiquitination (Li et al., 2001; Sionov et al., 2001). Apart from post-translational modifications such as phosphorylation, ubiquitination and acetylation, several proteins like ING1b, pRb, and MdmX inhibit p53-MdM2 interaction by directly
binding to p53 or MdM2 (Hsieh et al., 2001; Kamijo et al., 1998; Leung et al., 2002; Sharp et al., 1999). Oncogenes such as Myc and E1A are thought to activate p53 through a p19 alternative reading frame (p19ARF) (Seavey et al., 1999; Tolbert et al., 2002). The predominant regulator of MdM2 is p19ARF, a protein derived from the INK4a/ARF locus. This is located in nucleoli, where it promotes p53 accumulation by binding to and sequestering MdM2 (Sherr, 1998).

P53 stabilisation may also occur through MdM2-independent mechanisms. Caplain1, β-catenin and JNK have been shown to stabilise p53 in MdM2–null cell lines (Damalas et al., 1999; Fuchs et al., 1998; Kubbutat and Vousden, 1997). Protein Sin3a was shown to bind to p53 on the promoters of genes targeted for repression in response to DNA damage. This interaction stabilises p53 independent of MdM2 extended promoter association (Zilfou et al., 2001). Reduced nicotinamide adenine dinucleotide (NADH) quinone oxidoreductase 1 (NQO1) stabilises p53 by an MdM2 and ubiquitin independent mechanism. NQO1 is a unique oxidative stress specific pathway for p53 stabilisation (Asher et al., 2001; Asher et al., 2002).

Apart from p53 stabilisation, transcriptional activation of p53 is essential for initiating an early response to genotoxic stress. P53 regulates transcription of many down stream targets. However, the initial activation of its function as a transcription factor is the key for its ability to drive particular downstream pathways. Many residues at both the amino and carboxy terminus of p53 are phosphorylated in response to genotoxic stress. Phosphorylation of Ser46 is important in regulating UV induced apoptosis through the activation of gene p53AIP1. p53DINPl is also seems to be regulating Ser46 phosphorylation in response to genotoxic stress (Oda, 2000). Homeodomain-interacting protein kinase-2 (HIPK2) specifically phosphorylates Ser46 in response to UV radiation and distinctively drives
an apoptotic response (D'Orazi et al., 2002; Hoffmann et al., 2002). Ser392 at carboxy terminus is phosphorylated in response to UV radiation by complex containing CK2, hSpt16 and SSRP1 (Keller et al., 2001).

A unique combination of phosphorylated residues could serve as a framework for further post-translational modifications. There is evidence to link the importance of post-translational modifications with p53 transcriptional activation in response to genotoxic stress. Pin1, a propyl isomerase, binds to p53 and stimulates its transcriptional activity. This is due to conformational changes induced by Pin 1 (Zacchi et al., 2002; Zheng et al., 2002). Acetylation was shown to be an important modification of histones that correlated with increased transcriptional activity. CBP/p300, a protein with histone acetyl transferase activity is a co-activator of p53 and regulates its transcriptional as well as biological function in vivo. The functional consequences of acetylation are diverse and include DNA binding, enhancement of stability, and changes in protein-protein interactions (Avantaggiati et al., 1997; Gu et al., 1997; Lill et al., 1997). P53 is particularly acetylated at multiple lysine residues (Lys370, Lys371, Lys 372, Lys381 and Lys382) of the carboxy-terminal regulatory domain by CBP/p300 and, to a lesser extent, Lys320 by p300/CBP associated factor (PCAF). These acetylation sites are particularly important for ubiquitination and subsequent degradation of p53 by Mdm2 (Appella and Anderson, 2001).

1.1.6 Alterations of p53 – Carcinogen finger prints

P53 was found to be inactivated in almost 50% of human cancers (Hainut et al., 2000). The occurrence of missense mutations, deletions, or nonsense mutations of the gene prevents the protein from oligomerising and forming tetrameric complexes that can bind specific DNA sequences (Hussain and Harris, 1998). The frequency of p53
somatic mutations in sporadic human cancers varies 20% to 60% among types and can be over 80% in histological subtypes. These mutations were also found to be inherited in the families with a predisposition to multiple cancers such as in the Li-Fraumeni syndrome (Malkin, 1994). 87% of the p53 somatic mutations are missense mutations that lead to amino acid substitutions in proteins thus altering protein conformation. Most of the mutations (90%) identified to date have been observed in DNA binding domain and mainly effect amino acids of highly conserved regions (Hainut et al., 2000). The studies of the nature of p53 mutations and their distribution along the coding sequence have allowed the identification of specific mutation spectrum, revealing clues to the mechanisms of mutations. Some of these ‘mutagen fingerprints’ are well characterised and have been linked to exposure to various environmental mutagens and endogenous biological factors using epidemiological and experimental evidence. Well characterised and extensively documented examples are (1) the association of GC→TA transversions in lung cancer with tobacco smoke. (2) the link between dietary exposure to aflatoxin B and GC→TA transversions at codon 249 (Arg→Ser) in hepatocellular carcinomas (HCC) and (3) the strong correlation between CC→TT tandem dipyrimidine transition mutations in skin cancer and exposure to sunlight (Greenblatt et al., 1994; Hainaut and Holstein, 2000; Hollstein et al., 1998; Hussain and Harris, 1999).

Lung cancers have been strongly associated with tobacco smoking (Phillips, 1996). Residues most frequently mutated in the p53 gene of lung from smoking include codons 154, 156, 157, 158, 159, 237, 245, 248 and 273. Mutations consist predominantly of GC→TA transversions (Hernandez-Boussard and Hainut, 1998; Pfeifer and Denissenko, 1998; Smith et al., 2000). Codon 157 was considered hotspot specific for lung cancer. However, many studies reported that 157
is equally important in lung and non-lung cancers (Rodin and Rodin, 2000). Mutations found in codon 248 and 273 are Quiet common in other human malignancies except for the fact that the mutations found at these codons are exclusively GC→AT transitions than GC→TA transversions observed in lung cancer. Polycyclic aromatic hydrocarbons such as benzo[α]pyrene are the mutagens found in cigarette smoke. It was reported that p53 hotspot mutation sites correlated with the preferential adduct formation sites after exposure of human bronchial epithelial cells to the tobacco smoke metabolite benzo[α]pyrene diol epoxide (Denissenko et al., 1996).

The high prevalence of G→T transversions affecting the third nucleotide of codon 249 in heptaocellular carcinoma (HCC) has been distinctively associated with aflatoxin B exposure (Hsu et al., 1991). P53 mutation spectra obtained from HCC subjects from different regions of the world have been reported to vary widely (Montesano et al., 1997). In areas of high aflatoxin B exposure such as Qidong (China) and Mozambique, the frequency of GC→TA mutations in particular at codon 249 clearly dominates the spectrum (~ 90%). In contrast, the HCC related p53 mutation spectrum data from European countries and USA with little or no exposure to aflatoxin B have low prevalence of GC→TA mutations (~10%). Similar associations have been established between UV irradiation and development of human skin cancer using experimental and epidemiological evidence. Mutations found in p53 gene of non-melanoma skin cancer (NMSC) subjects exhibited a high frequency of C→T transitions, including tandem CC→TT mutations according to IARC p53 database. The mutations correlate with mutagenic effects of UV light via photoproducts such as cyclobutane pyrimidine dimers, which if not repaired, result in CC→TT tandem mutations (Brash et al., 1996; Greenblatt et al., 1994).
Gastro-intestinal cancer is the second most common cancer in the world. Current models suggest that human gastric carcinogenesis is a multistep and multifactorial process accompanied by an accumulation of multiple genetic alterations. The most common genetic alteration in gastric cancer involves the p53 gene. p53 alterations represent an early event in the intestinal type and a late event related to progression in the diffuse type gastric cancer. It is interesting to note that GC→AT transitions are common mutations in p53 and are specifically induced by alkylating agents, N-nitroso compounds (NOC), which are involved in the pathogenesis of gastro-intestinal cancer (Fedriga et al., 2000). The endogenous formation of NOC's derives from the interaction of amino compounds such as amino acids and nitrosating agents such as nitrates and nitrites. Many epidemiological case-control studies have supported this hypothesis, which showed that a major risk for stomach cancer is present in meat, nitrite and nitrate-rich diet. Alkylating NOC's are suspected of directly causing GC→AT transitions of the p53 gene and of mediating non-CpG site mutations, where the NO mediated deamination of 5-methylcytosine to give thymine may be important. This DNA methylation by alkylating NOC's causing GC→AT transition is also consistent with the role of alkylation of O6-position of 2-deoxyguanosine (Fedriga et al., 2000). It was reported that NO induces mutations in human cells. The p53 mutations observed in colon cancer could be induced by NO-related damage of thymine while those reported in liver and lung cancer could be induced by NO via depurination (Nguyen et al., 1992).

Hence, it would be interesting to discuss (1) the mechanisms of reaction of alkylating agents with DNA and relevance of specific alkyl adducts in induction of mutations. (2) The type of NOC’s and the mechanisms by which NOC compounds are formed endogenously. (3) The different sources through which
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humans are exposed to NOC. (4) The etiological role of NOC in the incidence of gastric-intestinal cancers? In this, various epidemiological studies, which have shown NOC is responsible for gastric and other human cancers will be discussed.
1.2 Reaction of Alkylating Agents with DNA

Alkylating agents react with the nucleophilic nitrogen and oxygen centres in DNA and at the oxygen atoms of the phosphate internucleotide linkage. They are at least 12 sites in DNA that can be targeted by alkylating agents [Table 1.1]. The specificity of reactions at different centres depend on factors such as the nucleophilicity of the DNA site, reactivity of the electrophilic species as well as steric effects i.e. accessibility.

**Sn1 mechanism:** *e.g.* methyl diazonium ion

\[
\begin{align*}
[R^- Y] & \xrightarrow{\text{Slow}} [R^+ + Y^-] \\
[R^+ + X^-] & \xrightarrow{\text{Fast}} RX
\end{align*}
\]

**Sn2 mechanism:** *e.g.* methyl methanesulphonate ion (MMS)

\[
X^- + [RY] \xleftrightarrow{} [XY^+] \xrightarrow{} X^+ + Y^- + RX
\]

*Figure 1.4: Sn1 and Sn2 types of nucleophilic substitutions. RY- alkylating agent/ electrophile (R - alkyl group, Y - leaving group) R^+ - (electrophile) carbocation, X^- - nucleophilic site in DNA, RX - alkylated product, MMS - methyl methanesulphonate*

Ring nitrogen’s such as N3 and N7 of adenine and guanine are the most nucleophilic sites in DNA whereas the exocyclic base oxygens are less nucleophilic centres (Beranek, 1990; Saffhill et al., 1985). Each alkylating agent produces a different spectrum of adducts and can react via either Sn1 or Sn2 mechanisms [Figure 1.4].
Nucleophilic substitution involving the Sn2 pathway is a bimolecular reaction, which is highly dependent on steric accessibility. During this type of substitution a transition complex is formed which results from the attack of electrophile on the nucleophilic centre of DNA. An alkylated product is formed upon the release of leaving group (Ehrenberg and Osterman-Golkar, 1980). In contrast, Sn1 reactions are unimolecular and follow in general first order kinetics. The rate of reaction is dependent on the formation of a reactive electrophilic intermediate called carbocation. The carbocation subsequently reacts with nucleophilic site in DNA thereby forming a covalently bonded DNA adduct (Beranek, 1990).

<table>
<thead>
<tr>
<th>Type of DNA base</th>
<th>Alkylation Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>N1- N3- N7-</td>
</tr>
<tr>
<td>Guanine</td>
<td>N3- N7- O6-</td>
</tr>
<tr>
<td>Cytosine</td>
<td>N3- O2-</td>
</tr>
<tr>
<td>Thymine</td>
<td>N3- O2- O4-</td>
</tr>
<tr>
<td>Internucleotide</td>
<td></td>
</tr>
<tr>
<td>Phosphate Chains</td>
<td>Phosphotriesters</td>
</tr>
</tbody>
</table>

Table 1.1: Alkylation sites in DNA

Less reactive, weak electrophiles such as methylmethanesulphonate (MMS) usually react via Sn2 mechanisms resulting predominantly in alkylation of DNA sites with relatively high nucleophilicity. Hence, high amount of adducts are formed mainly at nitrogen centres with very little reaction at oxygen sites.
In contrast, highly reactive electrophiles such as methylidiazonium ion tend to react via Sn1 mechanism. Irrespective of the nucleophilicity of DNA sites, highly reactive electrophiles generate a broad spectrum of adducts with alkylation occurring at nitrogen and oxygen sites [Table 1.2]. Alkylating agents such as N-methyl-N-nitrosourea (NMU), N-ethyl-N'-nitrosourea (ENU) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) follow Sn1 mechanism whereas MMS and DMS (dimethylnethanesulphonate) follow the Sn2 reaction pathway [Beranek (1990)]. However, there is some evidence that the apparent Sn1 mechanisms followed by alkylidiazonium ion have some Sn2 character in them. This is due to the highly

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Percentage of Total Alkylation by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MNU</td>
</tr>
<tr>
<td>N1-alkyladenine</td>
<td>0.7-1.3</td>
</tr>
<tr>
<td>N3-alkyladenine</td>
<td>8.0-9.0</td>
</tr>
<tr>
<td>N7-alkyladenine</td>
<td>0.8-2.0</td>
</tr>
<tr>
<td>N3-alkylguanine</td>
<td>0.6-1.9</td>
</tr>
<tr>
<td>N7-alkylguanine</td>
<td>65.0-70.0</td>
</tr>
<tr>
<td>O6-alkylguanine</td>
<td>5.9-8.2</td>
</tr>
<tr>
<td>N3-alkylcytosine</td>
<td>0.06-0.6</td>
</tr>
<tr>
<td>O2-alkylcytosine</td>
<td>0.1</td>
</tr>
<tr>
<td>N3-alkythymine</td>
<td>0.1-0.3</td>
</tr>
<tr>
<td>O2-alkythymine</td>
<td>0.1-0.3</td>
</tr>
<tr>
<td>O4-alkylcytosine</td>
<td>0.1-0.7</td>
</tr>
<tr>
<td>Phosphotriesters</td>
<td>12.0-17.0</td>
</tr>
</tbody>
</table>

Table 1.2: In vitro alkylation patterns of DNA expressed as a percentage of total alkylation [Beranek (1990)]. MNU- N-methyl-N-nitrosourea, ENU- N-ethyl-N-nitrosourea, MNNG- N'-methyl-N'-nitro-N-nitrosoguanidine, MMS - methyl methanesulphonate, DMS - dimethylsulphate, n.d. not detected or below limits of detection, dash indicates data not reported
reactive nature of the alkyldiazonium ion, which has an extremely stable leaving group — nitrogen.

These reactions can also be explained based on Swain-Scott relationship describing the reactivity of the alkylating agent with nucleophiles (Swain and Scott, 1953). Reagents with a low Swain-Scott constant tend to react extensively with less nucleophilic centres (e.g. O6 - position of guanine). In contrast, alkylating agents such as MMS with a higher constant tend to react with the more nucleophilic centres (e.g. N7 of guanine) (Vogel and Nivard, 1994). In fact, Sn1 and Sn2 mechanisms represent extremes for DNA alkylating agents. There is evidence that the methyldiazonium ion CH$_3$N$_2^+$ reacts with via the Sn2 (and not Sn1) pathway. This ion is, however, so reactive that it shows little discrimination between the nitrogen and oxygen centres in DNA (Loechler, 1994). The degree, to which DNA is alkylated and the relative quantity of the various adducts formed depend upon the type of alkylating agent and the nature of alkyl groups. The potential to react with oxygen atoms enhances the relative mutagenicity and carcinogenicity of various alkylating agents (Saffhill, 1984), thus explaining the increased mutagenicity reported for N-nitroso compounds compared to alkylmethylsulphonates and N-nitroso-N-ethylurea compared to its methyl analogue.

1.2.1 Relevance of Certain Alkyl DNA adducts to Mutation Induction

The DNA adducts formed at different sites of DNA are not equally mutagenic. It is necessary to distinguish between sites that are generally more susceptible to alkylation and those that that are biologically more significant. In general, DNA adducts at sites involving hydrogen bonding (such as exocyclic oxygen atoms) are more likely to induce mutations and to result in misincorporation during DNA
replication than those centres, which are not involved, such as N3 and N7 of guanine and adenine. N7-alkylguanine is the predominant adduct formed by alkylating agents, but the mutagenic, carcinogenic and cytotoxic potential of this adduct is considered to be of minor importance (Pieper, 1998). Interestingly N3 and N7-alkylpurines readily depurinate due to the labile nature of the glycosidic bond there by resulting in the formation of biologically significant apurinic sites (Pieper, 1998; Saffhill et al., 1985). Depurination of N3-methyladenine, N3- and N7- methylguanine is further facilitated by DNA glycosylases (Loeb, 1986). Apurinic sites lead to the preferential misincorporation of adenine residues opposite to these lesions during DNA replication resulting in GC→TA and AT→TA transversion mutations in the case of N7-methylguanine and N3-methyladenine respectively (Loeb, 1986).

DNA adducts formed at oxygen sites such as O^6- of guanine and O^2- and O^4- of thymine and O^2- of cytosine are more likely to be promutagenic than those formed at nitrogen centres (Saffhill et al., 1985; Singer, 1986; Singer and Essigman, 1991). O^4-methylthymine and O^6-methylguanine are the major promutagenic lesions formed by alkylating agents. Many in vitro and in vivo studies on O^4-methylthymine demonstrated that this adduct mispairs with guanine during replication [Figure 1.5]. This stable mispair induces AT→GC transition mutations (Preston et al., 1986). This mutation is consistent with the mutagenic and carcinogenic potential of alkylating agents that form O^4-methylthymine adducts. Although O^4-methylthymine adduct is formed to a lesser extent than O^6-methylguanine, the contribution of this adduct to the mutagenesis of alkylating agents is still of interest (Singer, 1986).
The mutagenic potential of $O^6$-alkylguanine adducts was first suggested by Loveless in 1969 (Loveless, 1969). Many studies demonstrated the relationship between persistence of $O^6$-alkylguanine adducts and alkylation induced mutagenesis (Frei et al., 1978; Goth and Rajewsky, 1974; Loveless, 1969; Richardson and Richardson, 1990; Samson and Cairns, 1977; Swann and Magee, 1968). The mispairing of $O^6$-methylguanine with thymine during DNA replication results exclusively in GC→AT transition mutations [Figure 1.5] (Loechler et al., 1984). Because of the specific mutagenic properties of NMU (G→A transitions) it has been possible to examine whether oncogenes can be the target of chemical carcinogens. It was demonstrated that ras oncogenes present in NMU induced mammary carcinomas in rats are activated by GC→AT transitions. This observation strongly supports the concept that
these mutations are caused by NMU directed methylation of the $O^6$-position of the 2'-deoxyguanosine (Zarbl et al., 1985). This type of mutation is also common in human colorectal cancer cases expressing ras mutations (Bos, 1989).

### 1.2.2 Sequence specificity and DNA Repair of $O$-Alkylated Bases

Apart from nature of the alkylating agents, DNA adduct formation is also influenced by the sequence context of the DNA, as the reaction of electrophiles with nucleophilic sites in DNA can be affected by bases neighbouring the target base (Richardson and Richardson, 1990). A strong preference for GC→AT transition mutations at sites preceded 5' by a purine base (5'-PuG-3') by Sn1 agents that act via an alkylidiazonium ion was observed in *E.coli*. However, this preference was not observed for Sn2 alkylating agents such as alkyl sulphates and alkyl alkanesulphonates (Glickman et al., 1987). The GC→AT transition induction is nine and five times at G preceded 5' by guanine or adenine respectively than those preceded by pyrimidine base (5'-PyG-3') (Bums et al., 1987). This sequence preference was explained by an influence of flanking base pairs on the molecular electrostatic potential of the $O^6$-position in guanine. This preference for 5'-PuG-3' over 5'-PyG-3' was supported by the observed alterations in the H-ras oncogene following NMU treatment (Zarbl, 1985). These observations were also extended to AT→GC transitions. Mutations induced by ENU via $O^4$-alkylthymine occur primarily at 5'-PuT-3' sites. This suggests that the distribution of mutations by this adduct is influenced by the same base context that influences $O^6$-alkylguanine directed mutations (Burns et al., 1988; Dolan et al., 1988). Many DNA repair studies demonstrated that $O^6$-alkylguanine and $O^4$-alkylthymine adducts flanked by a GC base pair are less efficiently removed by excision repair.
mechanisms than those flanked by AT base-pair (Burns et al., 1988; Horsfall et al., 1990).

DNA repair capability varies with the type of species, cell and organ, explaining in part the sensitivity of certain cells and organs to tumourigenesis. There are three main repair mechanisms existing for adducts generated by alkylating agents: (1) direct reversal of the modification by removal of only the altered group (2) Oxidative delakylation (3) base/nucleotide excision repair (Singer and Hang., 1997). The DNA adducts formed at the \( O^6 \)- position of the guanine are predominantly repaired by an alkyltransferase protein, \( O^6 \)-alkylguanine-DNA-alkyltransferase (ATase). This repair enzyme catalyses the transfer of alkyl group to the sulfhydryl group present on the cysteine residue within protein itself. The DNA sequence is therefore repaired in a single step. The cysteine acceptor site of the repair enzyme is not regenerated in this process (Kyrtopoulos, 1998; Lindahl, 1982; Pegg, 2000). Hence, the limiting factor for this repair process is the amount of repair enzyme, \( O^6 \)-alkylguanine-DNA-alkyltransferase present. The amount of this repair enzyme present is tissue and organ specific. Therefore, the tissues with less amount of this enzyme activity are more likely to form tumours (Karran and Lindahl, 1985; Pegg, 1984). The alkylated form of this enzyme is unstable in mammalian cells and degrades rapidly (Pegg and Byers, 1992). Currently there is no evidence of repair enzymes from eukaryotic sources that are able to remove \( O \)-alkylpyrimidines i.e., \( O^2 \)- and \( O^4 \)-alkythymine and \( O^2 \)-alkylcytosine efficiently (Singer, 1986). However, the \textit{E.coli} AGT protein has been reported to repair \( O^4 \)-alkythymine very efficiently using the same acceptor site as for the repair of \( O^6 \)-methylguanine, whereas the human ATase enzyme appears to be very poor in its function (Pegg, 2000).
Organisms have evolved a variety of mechanisms to repair alkylation damage. Recently, it was discovered that a family of proteins called the alk proteins could directly repair some of the alkylation DNA base damage. *E. coli* upon receiving a methyl group from the damaged DNA turns on transcriptional activator Ada protein, which in turn activates its own expression and that of *alkA, alkB* and *aidB* genes. The upregulated Ada is a bifunctional protein, which uses N-terminal Cys38 residue to remove methyl group from S\textsubscript{p}-methyl-phosphotriester and C-terminal Cys321 residue to remove a methyl adduct from both O\textsuperscript{6}-methylguanine and O\textsuperscript{4}-methylthymine (Mishina and He, 2006). *alkA* is a glycosylase that cleaves methylated bases from DNA and performs first step of well-known base excision repair of base lesions. 3-methyladenine is an abundant alkylated lesion generated by both Sn1 and Sn2 alkylating agents is preferentially repaired by *alkA* protein. It is a strongly cytotoxic base because its methyl group protrudes into the minor groove of DNA double helix, where it can block DNA replication and transcription. *alkA* protein has broad specificity. It also releases minor products such as O\textsuperscript{2}-methylcytosine, O\textsuperscript{2}-methylthymine generated by Sn1 agents as well as 8-methylguanine lesion generated by methyl radicals. *alkA* also liberates altered adenines such as ethenoadenine generated as a consequence of DNA exposure to lipid peroxidation products, hypoxanthine and uracil generated by active oxygen (Sedgwick and Lindhal, 2002). *alkB* protein utilizes a mononuclear non-heme iron (II) and α-ketoglutarate as cofactor and cosubstrate. These proteins activate dioxygen and oxidise methyl group. The resulting oxidised product decomposes spontaneously in aqueous solution to give formaldehyde and the repaired base. *E. coli* *alkB* performs an unprecedented oxidative dealkylation repair of N\textsuperscript{1}-methyl-adenine and N\textsuperscript{3}-methylcytosine. It is now known that *alkB* gene is conserved from bacteria to human. The
first human homologue now called ABH1 is 52% similar and 23% identical to E.coli \textit{alkB}. However, the purified gene product of ABH1 showed no activity like \textit{alkB}. Two other homologues ABH2 and ABH3 were shown to function like \textit{alkB} and could complement \textit{E.coli} mutant phenotype. Both the products showed repair activities for N\textsuperscript{1}-methyl-adenine and N\textsuperscript{3}-methyl-cytosine. It has been shown that ABH2 prefers N\textsuperscript{1}-methyl-adenine and ABH3 prefers N\textsuperscript{3}-methyl-cytosine (Mishina et al., 2006; Mishina and He, 2006).

Even though, the simplest pathway is direct DNA repair, most types of DNA damage are recognised and fixed by complex repair mechanisms such as excision repair and recombination. Base excision repair targets are mispaired, deaminated, alkylated, oxidised and otherwise modified bases. Highly specific enzymes called DNA-N-glycosylase that hydrolyses the N-glycoside bond between the sugar-phosphate backbone and damaged residues leaving an abasic site implement through base excision repair. DNA glycosylases differ in substrate specificity and function. Most of them recognise a broad range of substrates, others are highly specific. Some glycosylases may possess AP lyase activity. In mammals after damaged DNA has been excised, two alternate repair pathways are employed. In the case of short patch repair, a 5\textsuperscript{'}-AP endonuclease and deoxyribophosphodiesterase introduced a single nucleotide gap to the damaged strand, which is then reconstituted with DNA polymerase \(\beta\) and DNA ligase III. In the case of long patch repair, DNA segments 2-13 bases long are replaced around the AP site. DNA is resynthesised with polymerases \(\delta, \epsilon\) or \(\beta\). The synthesis is associated with creating a flap of "old" DNA, which is further removed by DNAse. DNA ligase completes the process of reinstating the phosphodiester bond. Alkylated bases commonly repaired by DNA-N-
glycosylases are 3-methyladenine, 7-methyladenine and 3-methylguanine (Vasilenko and Nevinsky, 2003).

Nucleotide excision repair system repairs damage in transcribed genes, therefore, it is often referred to as transcription-coupled repair (TCR). However, NER repairs damage regardless of its locations in the genome and current phase of cell cycle. Primary substrates of this type of repair are UV light-induced pyrimidine dimers, as well as chemical adducts and interstrand DNA cross-links. In E. coli, the NER pathway involves three enzymes UvrA, UvrB and UvrC. Eukaryotes from yeast to human do not have UvrABC homologues but, nevertheless, NER mechanisms in pro and eukaryotes are strikingly similar. In human, mutations in NER pathway genes are associated with disorders. Among them is Xeroderma Pigmentosum (XP). Mutations in any of the seven genes (XPA-XPG) eventuate in severe skin sensitivity to UV exposure (Vasilenko and Nevinsky, 2003).
1.3 What are NOC?

NOC stands for $N$-nitroso compounds, a class of carcinogens that have attracted much interest since $N$-nitrosodimethylamine (NDMA) was found to be a liver carcinogen in rats by Magee and Barnes in the mid 1950’s (Magee and Barnes, 1967)]. They are many types of nitroso compounds existing, but from carcinogenic point of view nitrosamines, nitrosamides and nitrosoureas are important. $N$-nitrosamides and $N$-nitrosoamines are stable components derived from parent nitrogen precursors by replacement of a proton by the nitroso group [Figure 1.6] (Shuker, 1988).

\[ \begin{align*}
\text{R'} &= \text{H} & \text{Primary amine} & \text{Primary nitrosamine (unstable)} \\
\text{R'} &= \text{alkyl} & \text{Secondary amine} & \text{dialkynitrosamines (metabolism required)} \\
\text{R'} &= \text{acyl} & \text{amide} & \text{nitrosamides (spontaneous decomposition)}
\end{align*} \]

Figure 1.6: Summary of the main $N$-nitrosation pathways giving rise to $N$-nitroso compounds (Shuker, 2000).

Humans are exposed to preformed NOC as well as to endogenously formed NOC. The \textit{in vivo} formation of NOC can occur by three different ways, they are as follows.

1. Acid-catalysed
2. Enzymatic reactions mediated by bacteria, virus and parasites \(\text{NO mediated}\)
3. Inflammation
Consequently, endogenous nitrosation may occur at various sites of the body such as the oral cavity, stomach, urinary bladder, lungs, and other sites of infection and inflammation. Indirect evidence for endogenous nitrosation comes from in vitro systems where background levels of DNA alkylation appear to be related to nitrosation of amino acids (Sedgwick, 1997). NOC form reactive DNA-alkylating agents upon spontaneous decomposition (diazoalkanes, nitrosoureas, nitrosoamides and related compounds) or after oxidation (dialkynitrosamines). The intermediate, probably an alkyl-diazonium ion, reacts with all of the nucleophilic centres in DNA [Figure 1.7] (Singer and Gumberger, 1983). Most interesting are $O^6$-alkylguanine and $O^4$-alkylthymine, since these are promutagenic modifications leading to misincorporation of opposing bases on DNA replication (Singer and Essigmann, 1991). There have been a number of reports associating elevated levels of this $O^6$-methyl...
adduct with increased risk of cancer (The Eurogast study Group, 1994; Umbenhauer et al., 1985).

Umbenhauer (Umbenhauer et al., 1985) had used radioimmuno assay techniques to detect $O^6$-MedG and $O^6$-ethyl-2'-deoxyguanosine ($O^6$-EtdG) in human tissues by using monoclonal antibodies against the adducts. Their analysis comprised 37 human tissue specimens derived from patients of People Republic of China, who underwent surgery for cancer of the oesophagus. Twelve tissues samples obtained from hospitals in Europe were used as controls. Seventeen of the samples obtained from china showed a level of $O^6$-MedG ranging from 15 -50 fmol/mg. Ten of the samples showed higher levels up to 160 fmol/mg and the remaining samples were below the level of detection. The tissue samples from Europe showed levels below 45fmol/mg $O^6$-MedG adduct of which seven being below the limit of detection. $O^6$-EtdG was not detected in any of the above samples. The activity of $O^6$-alkylguanine-DNA-alkyltransferase in tissue samples from china ranged from 190 – 326 fmol of $O^6$-MedG removed from mg of protein.

1.3.1 Mechanisms of Endogenous Nitrosation

1.3.1.1 Acid Catalysed Nitrosation

Nitrosation of amines and amides to form carcinogenic N-nitroso compounds were studied in detail by Ridd in 1961 (Ridd, 1961). To affect nitrosation, nitrite must be acidified to form nitrous acid (HNO$_2$). Since, this reaction requires acidification, it occurs in vivo mainly in the acidic stomach. This dimerizes with loss of water to form N$_2$O$_3$, which reacts with amines to form NOC. Nitrous acid can also be protonated to form H$_2$NO$_2^+$, which preferentially reacts with amides to form nitrosoamides (Mirvish, 1995).
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Reactions:

**Nitrosation of Secondary amines**

\[
\text{NaNO}_2 + \text{H}^+ \longrightarrow \text{HNO}_2 + \text{Na}^+ \\
2\text{HNO}_2 \longrightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O} \\
\text{N}_2\text{O}_3 + \text{HNR}_1\text{R}_2 \longrightarrow (\text{NO})_x \text{NR}_1\text{R}_2 + \text{HNO}_2
\]

**Nitrosation of Amides**

\[
\text{HNO}_2 + \text{H}^+ \longrightarrow \text{H}_2\text{NO}_2^+ \\
\text{H}_2\text{NO}_2^+ + \text{RNHCOR}' \longrightarrow \text{RN(NO)COR}' + \text{H}_2\text{O} + \text{H}^+
\]

It has also been shown that nitrosation also occurs at both neutral and basic pH in the presence of NO' and O_2. Challis and Kyrtopolous (1978) demonstrated that N_2O_3 and N_2O_4 formed from NO' and O_2 rapidly nitrosate amines, both at neutral and basic pH. The mechanism of nitrosation at neutral and basic conditions is nucleophilic attack on the nitrosyl nitrogen of \(\text{O} = \text{N} - \text{O} - \text{N} = \text{O}\) (N_2O_3) by the free amine (Challis and Kyrtopolous, 1978).

Reactions

**Formation of Nitrosating agents**

\[
2\text{NO}^- + \text{O}_2 \longrightarrow 2\text{NO}_2^- \\
2\text{NO}_2^- \longrightarrow \text{N}_2\text{O}_4 \longrightarrow \text{NO}_2^- + \text{NO}_3^- + 2\text{H}^+ \\
\text{NO}^- + \text{NO}_2^- \longrightarrow \text{N}_2\text{O}_3 \longrightarrow 2\text{NO}_2^- + 2\text{H}^+
\]

**Nitrosation of a secondary amine**

\[
\text{N}_2\text{O}_3 + \text{R}_2\text{NH} \longrightarrow \text{R}_2\text{N} - \text{N} = \text{O} + \text{HNO}_2
\]
Nitrosation at neutral and basic conditions can also be catalysed by carbonyl compounds. The catalytic effect depends upon the structure of carbonyl compounds. Keefer and Roller (1973) demonstrated for the first time that the secondary amines were easily nitrosated by nitrite ion in alkaline formaldehyde solution (Keefer and Roller, 1973). However, nitrosation at neutral and basic pH is not significant with respect to endogenous nitrosation, since $N_2O_3$ was thought to be formed primarily in acidic stomach (Liu and Hotchkiss, 1995).

1.3.1.2 NO - Mediated Endogenous Nitrosation

Several human cancers have been associated with chronic bacterial, viral and parasitic infections. NO$^\cdot$ levels are elevated in these infections. Long-term exposure to elevated NO$^\cdot$ in cells could have potential genotoxic effects on hosts. They are at least three mechanisms by which intracellular elevated NO$^\cdot$ could exert genotoxic effects after reacting with $O_2$. These include

1. Formation of carcinogenic $N$-nitroso compounds
2. Direct deamination of DNA bases
3. Oxidation of DNA after formation of peroxynitrite and hydroxy radicals (Liu and Hotchkiss, 1995).

NO is synthesised from L-Arg through the L-Arg-NO$^\cdot$ pathway by NO$^\cdot$ synthase (NOS) with NADPH (Marletta, 1988; Marletta, 1993; Stuehr et al., 1989) and oxygen as co-substrates (Kwone et al., 1990; Leone et al., 1991). The enzyme has many features in common with P-450 enzymes. Three different forms of NOS are found existing, they are as follows

1. eNOS (present in the endothelium of blood vessels)
2. nNOS (present in neurons)
3. iNOS (present in immune system cells and hepatocytes) (Liu and Hotchkiss, 1995).

A. Formation of Nitrosating agents

\[
2\text{NO}^\cdot + \text{O}_2 \rightarrow 2\text{NO}_2^\cdot \\
2\text{NO}_2^\cdot \leftrightarrow \text{N}_2\text{O}_4 \rightarrow \text{NO}^- + \text{NO}_3^- + 2\text{H}^+ \\
\text{NO}^\cdot + \text{NO}_2^\cdot \leftrightarrow \text{N}_2\text{O}_3 \rightarrow 2\text{NO}_2^- + 2\text{H}^+
\]

B. Nitrosation of a secondary amine

\[
\text{N}_2\text{O}_3 + \text{R}_2\text{NH} \rightarrow \text{R}_2\text{N} = \text{O} + \text{HNO}_2
\]

C. Deamination of primary amine

\[
\text{N}_2\text{O}_3 + \text{R-NH}_2 \rightarrow \text{R}^+ + \text{N}_2 + \text{NO}_2^- + \text{H}_2\text{O}
\]

D. Hydroxy radical production

\[
\text{NO}^\cdot + \text{O}_2^\cdot \rightarrow \text{ONO}^\cdot \\
\text{ONO}^\cdot + \text{H}^+ \rightarrow \text{ONO}^- \\
\text{ONO}^- \rightarrow \text{HO}^\cdot + \text{NO}_2^\cdot
\]

Both eNOS and nNOS are constitutive and Ca\(^{2+}\)/Calmodulin dependent. Agonists such as acetylcholine and bradykinin that elevate intracellular Ca\(^{2+}\) alter the conformation of calmodulin, which binds to NOS inducing NO synthesis from L-Arg (Liu and Hotchkiss, 1995). iNOS is an inducible Ca\(^{2+}\)/calmodulin independent flavoprotein in which tetrahydrobiopterin acts as a cofactor (Stuehr et al., 1991; Tayeh and Marletta, 1989).
Because NO\(^{\cdot}\) has an unpaired electron it is highly reactive in the gas phase with oxygen to form nitrosating agents (Liu and Hotchkiss, 1995). NO\(^{\cdot}\) reacts with molecular oxygen (O\(_2\)) to produce NO\(_2^{\cdot}\), which exists in equilibrium with the nitrosating agents N\(_2\)O\(_3\) and N\(_2\)O\(_4\) (Marletta, 1988). This occurs at both gaseous and aqueous phase, but it is first order reaction in the former (Wink, 1993) and second order reaction in the latter (Stamler et al., 1992). The formation of nitrosating agents N\(_2\)O\(_3\) and N\(_2\)O\(_4\) are favoured in solution compared to gaseous phase and the reaction is likely to be slow at physiological concentrations (Furchgott and Vanhoutte, 1989). Both N\(_2\)O\(_3\) and N\(_2\)O\(_4\) are capable of reacting with water to yield NO\(_2^{\cdot}\) and NO\(_3^{\cdot}\). NO\(_3^{\cdot}\) is the urinary excretory end product of NO (Liu and Hotchkiss, 1995).

There is substantial evidence that NO\(^{\cdot}\) synthesis and endogenous nitrosation are elevated because of acute inflammation and chronic infection (Bartsch et al., 1989; Hotchkiss et al., 1992). In 1987, it was reported that bacterial urinary tract infections in humans cause increased urinary excretion of nitrosoamino acid and nitrosamines (Ohshima et al., 1987). Tricker reported that patients having bladder cancer due to infection with schistosoma haematobium infection and patients with urinary diversions excreted more NO\(_2^{\cdot}\) and NO\(_3^{\cdot}\) and N-nitroso aminoacids (Tricker et al., 1989a; Tricker et al., 1989b). It was found that bacteria can catalyse nitrosation. This activity is linked to nitrate reductase in non-denitrifiers and to nitrite reductase in denitrifiers (Suziki and Mituoka, 1984).

Humans infected with liver flukes have an elevated risk of cholangiocarcinoma. Srianujata observed that subjects with cholangiocarcinoma have elevated urinary NO\(_3^{\cdot}\) levels (Srianujata et al., 1987). A human study with controlled low NO\(_3^{\cdot}\) diet showed that subjects with \textit{O.viverini} liver fluke infections had increased amounts of salivary NO\(_2^{\cdot}\) and urinary and plasma NO\(_3^{\cdot}\). Ohshima found
that hamsters infected with the above liver fluke excreted the increased amounts of NO$_3^-$ and N-nitrosoamino acid in urine (Ohshima and Bartsch, 1994). Woodchucks chronically infected with WHV have shown increased synthesis and excretion of NO$_3^-$ and N-nitrosodimethylamine (NMDA). Treatment of WHV infected animals with E. coli LPS demonstrated 15 fold increase in NO$_3^-$ excretion (Liu et al., 1991). The same results have been obtained from human studies in which subjects have chronic active hepatitis (Ohshima and Bartsch, 1994). Workers at the University of Pittsburgh have showed that hepatocytes formed NO$^-$ when treated with immunostimulants (Billiar et al., 1990a; Billiar et al., 1990b).

It has been shown that in cirrhosis patients there has been an increased background formation of N-nitroso amino acid (Bartsch et al., 1989)]. Liu et al., (1992) have demonstrated elevated NO$^-$ formation \textit{in vitro}. \textit{In vitro} and \textit{in vivo} data suggest that elevation of NO$^-$ synthesis may be a general and primary response to infections and inflammation (Liu and Hotchkiss, 1995).
1.4 Exposures to NOC

1.4.1 Tobacco products

Cigarette smoking causes 30% of all cancers in the USA and Western Europe. There are 55 carcinogens in cigarette smoke that have been evaluated by the International Agency for Research on Cancer. Tobacco specific nitrosamines such as N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polyaromatic hydrocarbons (PAH) contribute to the induction of cancer of the lung, oesophagus, pancreas, nasal and oral mucosa. The levels of NNK and PAHs in cigarette smoke are similar (Hoffmann and Hecht, 1990). However, NNK is the only compound that can induces lung tumours in all three rodent models, rats, mice and hamsters. The organospecificity of NNK for the lung is remarkable. It induces adenoma and adenocarcinoma, independent of the route of administration. It metabolises via α-hydroxylation to form intermediates that alkylate DNA bases with methyl and pyridyloxobutyl groups (Hecht, 1998). Hydroxylation of α-methylene carbon of NNK generates an unstable α-hydroxynitrosamine that spontaneously decomposes to methanediazohydroxide, which reacts with DNA to form methyl adducts such as O6-MedG and N7-MedG (Hecht, 1998). The similar pathway also generates diazonium ion is a pyridyloxobutylating agent. It reacts with DNA bases to form pyridyloxobutyl adducts (Hecht, 1998; Thomson et al., 2003; Wang et al., 2003). The quantitative studies on the pyridyloxobutyl adducts formed by the NNK reported that the most abundant adduct formed by NNK is 4-hydroxy-1-(3-pyridyl)-1-butanone (Sturla et al., 2005). Although, both α-hydroxylation pathways of NNK lead to the formation of DNA adducts and important to the tumorigenicity of NNK, a preponderance of experimental data in mice implicates α-methylene hydroxylation and subsequent formation of O6-methylguanine as crucial factors for NNK.
tumorigenecity (Jalas et al., 2003; Peterson et al, 2001). NNN induces oesophageal and nasal tumours in rats and is likely to play an important role in tobacco-related oesophageal cancer in smokers. (Hecht, 1998; Hoffman and Hecht, 1990). The association of snuff dipping with nasal cancer is due to NNN because the NNN and its NNN-N-oxide induce nasal squamous carcinoma in rats. NNN is metabolically activated by hydroxylation at 2' position causing the opening of ring. This result in formation of mutagenic pyridyloxobutyled-DNA adducts by NNN. $O^6$-pyridyloxobutyl-dG is one of the products of this pathway was found to be highly mutagenic in both bacterial and mammalian cells (Pauly et al., 2002; Wang et al., 2003). Analysis of human lung DNA demonstrated that levels of pyridyloxobutylated DNA are higher in lung cancer patients than in controls (Schlobe et al., 2002). Oral cancer is associated with chewing tobacco in USA and chewing quids along with betel nuts and tobacco in India (Mirvish, 1995). Among the PAHs, benzo(a)pyrene (Bap) is the most extensively studied compound, and its ability to induce lung tumours upon local administration and inhalation is well documented (Thyssen et al., 1981; Wolterbeek et al., 1981). When administered systemically, it causes lung tumours in mice, but not in rats (Culp et al., 1998). The major metabolic pathway of activation for BAP is conversion to its 7,8-diol-9,10-epoxides (BPDE), one of the four enantiomers is highly carcinogenic and reacts with DNA to form adducts with $N^2$ of deoxyguanosine (Hecht, 1996; Hecht, 1998). Carcinogenic PAHs such as Benzo(a)pyrene, 7, 12b-dimethylbenz(a)anthracene and dibenzo(a)pyrene have been shown to be metabolically activated by two major mechanisms, one electron oxidation to form radical cation intermediates and monooxygenation to form bay region diol epoxides. The adducts formed by these mechanisms can be either stable or depurinating (Todorovic et al., 2005). BaP is metabolically activated via a three-step
process. First, cytochromes P450 catalyze the formation of \((7R,8S)\)-epoxy-7,8-dihydrobenzo[a]pyrene(BaP-7,8-oxide). This is converted to \((7R,8R)\)-dihydroxy-7,8-dihydrobenzo[a]pyrene (BaP-7,8-diol), catalyzed by epoxide hydrolase. BaP-7,8-diol then undergoes another oxidation step, catalyzed by cytochromes P450 and other enzymes, producing mainly \((7R,8S)\)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE). Among the four possible 7,8-diol-9,10-epoxide isomers of BaP, BPDE is formed to the greatest extent in mammalian systems examined to date, and has high tumorigenic activity in murine models. BPDE reacts with DNA producing a major adduct at the \(N2\) position of deoxyguanosine (BPDE-\(N2\)-dG). Convincing evidence clearly documents the presence of this adducts in target tissues of animals treated with BaP. The mutagenicity of BPDE-\(N2\)-dG and its effects on DNA conformation have also been conclusively demonstrated (Boysen and Hecht, 2003).

1.4.2 Nitrite Cured Meat

Consumption of nitrite preserved meat and fish has been associated with childhood leukaemia and brain cancer, two of the most common cancers in children. Many studies have linked this childhood leukaemia and brain cancer with the consumption of hotdogs by pregnant mothers. Fried bacon formerly contains up to 100ppb of nitrosamines chiefly N-nitrosopyrrolidine (NPYR) and N-nitrosodimethylamine (DMN). Similar studies have indicated during frying and drying process of bacon, dehydration of creatine results in the formation of creatinine, which easily undergoes nitrosation to form methylurea (Mirvish, 1995). Hot dog consumption by children was linked with childhood leukaemia, with odd ratios of 9.5 and 95% confidence interval of 1.6-58 for \(\geq\)12 hot dogs/mo (Peterson et al., 1994). The levels of NOC measured in
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the gastrointestinal tracts of Sprague-Dawley rats fed with semipurified diet, 180g beef-pork hot dogs mixed with 820g diet or 180g sauteed beef mixed with 820g diet showed a 16-fold increase from stomach to proximal small intestine and 1.7 fold increase from distal colon to faeces. In addition, Swiss mice fed with semipurified diet, 180g beef-pork hot dogs mixed with 820g diet or 180g sauteed beef mixed with 820g diet have produced fecal NOC outputs of 3.5 – 7.0 (hotdogs) and 2.0 -2.9 (beef) times with respect to control groups. Overall, the results support the hypothesis that colonic NOC's are a cause of colon cancer (Mirvish, 2002; Mirvish, 2003). The consumption of nitrite cured meat by pregnant women was linked with brain cancer in their children in nine control studies (Bunin, 1998). A meta-analysis was performed examining the possible association of maternal intake of cured meat during pregnancy and risk of paediatric brain tumours. Analyzing child brain tumour risk by type of cured meat ingested showed that hot dog consumption increased child brain tumour risk by 33%, with a similar increase shown by frequent ingestion of sausage by 44% (Huncharek and Kupelnick, 2004). Despite the apparent risk, meat remains a nutritionally important component of most western diets (Truswell, 2001). Processed meat was also associated with a higher risk for type 2 diabetes, with a relative risk of 1.46 and a 95% confidence interval of 1.14-1.86 for consumption ≥ 5times/wk compared with less than once per month (Van Dam et al., 2002).

1.4.3 Alcohols and Beverages

Until 1979, beer contained about 5ppb of DMN. This is due to the reactions between tertiary amines hordenine and gramine in malted barley with nitrogen oxide present in natural gas kilns in which malted barley was dried. After 1980 DMN content was lowered by reducing flame temperature, by adding elemental sulphur to the flames or
by indirect heating of the malted barley (Mirvish, 1995). 1-Methylpyrene, a hepatocarcinogen, is metabolised via 1-hydroxymethapyrene (HMP) to a reactive sulphuric acid ester. These esters form either DNA adducts or conjugated with glutathione to form Methylpyrenyl mercapturic acid. It was reported that bioactivation of HMP in rats treated with 4-Methylpyrazole (alcohol dehydrogenase inhibitor), disulfiram (aldehyde dehydrogenase inhibitor) or ethanol (a substrate of alcohol dehydrogenases) caused increase in the levels of hepatic DNA adducts by 28, 3.8 and 15-fold, respectively (Ma et al., 2002). The effect of ethanol and acetaldehyde treatment on the removal of benzo(a)pyrene diol epoxide (BPDE)-DNA adducts in the immortalised human mammary epithelial cell line MCF-10F was examined. Cells treated with 15 and 25mM ethanol resulted in significantly higher half-life of BPDE-DNA adducts/Units DNA compared to controls. There is no particular trend observed for cells treated with 2.5 and 5.0μM acetaldehyde with respect to controls. In addition, cells treated with 25mM ethanol exhibited a 2-fold increase in 8-oxo-deoxyguanosine adducts compared to controls (Singletary, 2004). Alcoholic beverage consumption is causally related to an increased risk of cancer of the upper gastro-intestinal tract. Acetaldehyde causes sister chromatid exchanges and chromosomal aberrations in human cells. The best studied DNA adduct is N[2]-ethyl-2'-deoxyguanosine, which is increased in liver DNA obtained from ethanol treated rodents and white blood cells obtained from human alcohol abusers. However, carcinogenic relevance of this adduct is unclear. Recently it was reported that 1,N[2]-propano-2'-deoxyguanosine can also be formed from acetaldehyde in genotoxic and mutagenic effects of crotanaldehyde. This adduct can exist in two forms: a ring closed form or a ring opened aldehyde form. Ring-closed from is mutagenic. The aldehyde form can participate in the formation of secondary lesions, including DNA-protein cross-links and DNA
interstrand cross-links, which explain many of the genotoxic effects of the acetaldehyde (Brooks and Theruvathu, 2005) (Brooks and Theruvathu, 2006).

1.4.4 Drugs

A considerable fraction of drugs are theoretically nitrosable due to the presence of secondary or tertiary amines or N-substituted amines, amides or other groups which by reacting with nitrite in the gastric environment. When these drugs are administered chronically, high gastric levels of these drugs can occur and easily undergo nitrosation to give carcinogenic NOC. The yield of NOC not only depends on chemical structure of the drug but also on the drug-nitrite molar ratio, pH, temperature and reaction time. In many cases, the drug-nitrite interaction product is, in a foreseeable way, an already known NOC; for example, the NOC formed by several drugs is N-nitrosodimethylamine and N-nitrosodiethylamine. For other drugs, the reaction of the secondary amino group with nitrite yields the expected N-nitroso derivative; e.g., N-nitrosomethamphetamine, N-nitrosoephedrine, N-nitrosopropanolol and other N-nitroso β-blockers. Sometimes the reaction products formation is much more complex. IARC classifies N-nitrosodimethylamine, N-nitrosodiethylamine, N-methyl-N-nitrosourea and N-ethyl-N-nitrosourea as probably carcinogenic to human. Many drugs like chlordiazepoxide, a tranquiliser, antihistamines like methapyrilene, chlorpheniramine and diphenhydramine, alcohol deterrent disulfiram and the antibiotic oxytetracycline react with nitrite to give N-nitrosodiethylamine (DEN) and DMN. The tertiary amine analgesic, aminopyrine, is readily nitrosated to give DMN because of its unusual enamine structure. Piperazine is a cyclic secondary diamine, which undergoes nitrosation to form carcinogens, mono and dinitrosopiperazine (MNP and DNP) (Mirvish, 1995; Brambilla and Martelli, 2007). 3'-Azido-3'-deoxythymidine
(AZT, Zidovudine) has been effectively used for HIV infection treatment. It inhibits virus reproduction through virus reverse transcriptase inhibition. However, the side effects of this anti-retroviral drug might be cumulative, particularly in its effects on the patients DNA. As a nucleoside analogue AZT might incorporate in to hosts DNA, and then form DNA adducts. This may result in potential long-term risks of mutagenesis in AIDS patients who received therapy (Huang et al., 2004).

1.4.5 Industrial Exposure

Workers in certain industries are exposed to carcinogenic nitrosoamines. Workers in rubber industry are exposed to nitrosoamines such as DMN, DEN and N-nitrosomorpholine (NMOR), which are formed during vulcanisation process. There is an association between working in the rubber industry and cancer of the bladder, stomach, lung and leukaemia. The relationship between exposure to high concentrations of nitrosamines and cancer mortality among a cohort of rubber workers was examined. It was reported that nitrosamine exposure was significantly associated with increased mortality from cancers of the oesophagus and of the oral cavity and pharynx. A non-significant trend of increasing mortality was found for cancer of the prostrate and brain. No association was found between exposure to notrosamines and stomach cancer (Straif et al., 2000). Occupational nitrosamine exposure from a rubber vehicle seal curing operation were compared with peripheral blood concentrations of two nitrosamines related DNA adducts N7-methylguanine and O6-methylguanine. No significant association was found between nitrosamine exposure and N7-methylguanine. However, a positive association between nitrosamine exposure and O6-methylguanine adducts was found, linking nitrosamine exposure one-step closer to human cancers (Reh et al., 2000). (1-
Chloroethenyl)oxirane is a major mutagenic metabolite of chloroprene, an important large-scale petrochemical used in the manufacture of synthetic rubbers. It is reactive towards DNA forming wide variety of mutagenic DNA adducts such as N7-(3-chloro-2-hydroxy-3-buten-1-yl)-2'-deoxyguanosine, N1-(3-chloro-2-hydroxy-3-buten-1-yl)-2'-deoxyadenosine, N6-(3-chloro-2-hydroxy-3-buten-1-yl)-2'-deoxyadenosine N3-(3-chloro-2-hydroxy-3-buten-1-yl)-2'-deoxyuridine and N3-(3-chloro-2-hydroxy-3-buten-1-yl)-2'-deoxythymidine (Munter et al., 2002), (Munter et al., 2006). 1,3 - Butadiene is a high production volume chemical used in chemical industry. It is also ubiquitous environmental pollutant found in urban air, tobacco and car exhausts. It is metabolised to butadiene monoepoxide and diepoxyl butane and butadiene diolepoxide. These epoxides are genotoxic and alkylate DNA both in vitro and in vivo mainly at N7 position of guanine (Koivisto et al., 1999; Selzer and Elferra, 1999). Workers from leather tanneries are exposed to DMN, which is formed from DMSO used to remove hair (depilate) from the skins. Metal industries use metal working fluids contain N-nitrosodiethanolamine (NDELA) added as a rust retarder. NDELA penetrates the skin and lung so that people are exposed to it by direct contact and inhalation (Mirvish, 1995). Levels of 8-hydroxy-2'-deoxyguanosine in DNA of white blood cells of workers exposed to asbestos fibres were examined in Germany. For patients suffering from respiratory cancer, cancer of the gastrointestinal tract, mouth/pharynx/larynx and urogenital tract the mean DNA adduct levels is significantly higher (Marczynski et al., 2000). Chromium is corrosive, cytotoxic and carcinogenic for humans and can induce acute and chronic lung tissue toxicity. Chromium causes biochemical changes in the airways causing oxidative stress via hydrogen peroxide and Malondialdehyde (Caglieri et al., 2006)
Despite of a large store of accumulated knowledge about the modes of action of $N$-nitroso compounds (NOC), there was still a great uncertainty about the role of NOC in human cancer. For many years, it was thought that the main route of exposure to NOC was from external sources. Endogenous synthesis of NOC in animals was initially reported by Sander and Burkle in 1969 (Sander and Burkle, 1969), and in humans by Ohshima and Bartsch in 1981 (Ohshima and Bartsch, 1981). This opened up the possibility of completely different and possible major source of NOC. Since then evidence has accumulated suggesting that body fluids, particularly, gastric juice do contain NOC and that certain pathologies (e.g., chronic inflammation or bacterial overgrowths) increase the levels of NOC (Shuker, 2000).

An etiological model proposed by Correa in 1975 proposed a role for NOC in gastric cancer. It proposes that irritants and nutritional deficiencies contribute to the development of atrophic gastritis. This leads to the increase in gastric pH thus promoting bacterial growth. These bacteria promote the reduction of nitrate (from food and endogenous sources) to nitrite. In the absence of antioxidants (such as vitamin C and E, which inhibit nitrosation), this nitrite is available to combine with compounds from drugs and food to form NOC. Exposure of gastric mucosa to small doses of NOC over a prolonged period is postulated to be a key event in the carcinogenic process (Correa, 1992; Susan and Correa, 1989).

Experimental data from many epidemiological studies give supporting evidence for the above hypothesis. Bartsch et al used the N-nitrosoproline (NPRO) test to test Correa's model in subjects with pancreatic cancer. NPRO is an unusual nitrosamine in that it is resistant to metabolic activation and is excreted
unchanged in the urine. Furthermore, its precursor L-proline, a natural amino acid, is capable of being efficiently nitrosated under mildly acidic conditions where as more basic dialkylamines are protonated and are less reactive. Later it was reported that NPRO levels were high in subjects who lived in high cancer risk areas of the world (Bartsch et al., 1988; Bartsch et al., 1988; Bartsch et al., 1992).

![Graph showing levels of total N-nitroso compounds (NOC) in fasting gastric juice at a wide range of pH values (Xu and Reed, 1993).](image)

Figure 1.8: The graph showing levels of total N-nitroso compounds (NOC) in fasting gastric juice at a wide range of pH values (Xu and Reed, 1993).

Earlier studies have reported the presence of background levels of NPRO in human urine and in elegant series of animal experiments with stable isotope labelled NPRO and nitrite. Humans are exposed to nitrate from exogenous sources (food and water) and endogenous sources (nitrate is the end product of the in vivo enzymatic formation of oxides of nitrogen). Chronic diseases or infections such as hepatitis can produce elevated levels of nitrate. Both endogenously formed and environmentally derived nitrate are found in saliva and gastric fluid, where they can be reduced to nitrite.
Human exposure to exogenous and endogenous nitrate results in the formation of N-nitroso compounds in both stomach and other sites (Hotchkiss et al., 1985). Hotchkiss and colleagues showed that urinary NPRO was derived from both exogenous and endogenous sources as well as the mixed intermediate routes (Perciballi et al., 1989).

Further support for the Correa elaboration of hypothesis and the role of NOC in gastric cancer has led to the quantifying of NOC levels in the dynamic system of gastric contents by several groups. A well conducted and extensive series of observations by Xu and Reed demonstrated that total NOC concentrations varied as a function of intragastric pH with maximal values being found at either end of the scale from pH~2 to pH~8 [Figure 1.8]. It has been shown that precursor nitrogenous compounds can be converted into NOC under acidic conditions (via a nitrous mediated pathway) or at neutral or mildly alkaline conditions (via gaseous oxides of nitrogen of bacterial and from other sources). A significant relationship between NOC and intragastric pH was found in the high pH range 5.00 – 8.42 (r = 0.218, P < 0.01). However, in the low pH 1.13 – 4.99 there was no significant relationship between NOC and intragastric pH (r = -0.067, P > 0.05). Increased NOC concentrations were observed in samples at both low and high pH ranges. Mean NOC concentrations in gastric juice samples at pH 1.13 – 2.99 and 6.00 – 8.42 were significantly higher than those at pH 3.00 – 5.99 and those at pH 6.00 - 8.42 were also significantly higher than the NOC levels at pH 1.13 -2.99 (Xu and Reed, 1993).

*H. pylori* infection is very common, but only a small proportion of people infected with the bacterium develop gastric cancer. Such infection is therefore not sufficient, in itself, to cause gastric cancer. One possible explanation is that the infection induces predisposing pathological changes in the gastric mucosa,
such as atrophic gastritis and intestinal metaplasia, which in turn increase the risk of gastric cancer. In attempts to integrate the role of NOC, dietary factors and \textit{H. pylori} infection in gastric carcinogenesis, the first issue to be addressed is why the relative risk for \textit{H. pylori} infection is similar in gastric cancers of the diffuse and intestinal types. The epidemiological features of the two forms are quiet different; the intestinal type is believed to comprise the epidemic component of gastric cancer, whereas the risk of the diffuse type is stable across country and region. In addition, the risk of the
intestinal type, but not diffuse type, is related to chronic atrophic gastritis and metaplasia. The reasons for these differences are unknown. An integrated model was proposed by Yamaguchi and Kakizoe (Yamaguchi and Kakizoe, 2001) offering some possible explanations for the above inconsistencies [Figure 1.9].

One shared characteristic feature of diffuse and intestinal types as regards the effect of *H. pylori* infection is the enhancement of inflammatory responses, including epithelial-cell proliferation, infiltration of neutrophil and macrophages, and oxidative DNA damage. One possible explanation is that the inflammatory response directly increases the risk of developing the diffuse type of cancer, and the same response increases the risk of chronic atrophic gastritis and intestinal metaplasia in patients who ultimately develop intestinal-type gastric cancer. The fact that the incidence of diffuse-type gastric cancer does not differ by region suggests that the genetic susceptibility has a role in this type, as shown by the previously mentioned polymorphisms in the interleukin 1 gene cluster (El-Omar, 2000). In the intestinal type of gastric cancer, environmental factors other than *H. pylori* infection seem to play a part in the carcinogenic process. These factors may facilitate the development of atrophic gastritis and intestinal metaplasia, or alternatively they may increase endogenous nitrosation as result of ingestion of larger amounts of nitrate, nitrite, or secondary amines. The equal sex distribution of the diffuse type seems to reflect the equal sex distribution of *H. pylori* infection, whereas, the male dominance of the intestinal type could reflect the sex differences in some of the other factors. Exposure of N-nitroso compounds, which causes genetic events leading to cancer, probably facilitates advancement of chronic atrophic gastritis and intestinal metaplasia of adulthood. By contrast, in the diffuse type, genetic events probably occur at an earlier stage in the life, along with *H. pylori infection*. This
difference in timing of genetic events may explain the older age at onset of intestinal type. Two protective actions of fresh fruits and vegetables are likely: as antioxidants to protect the gastric epithelium from inflammatory responses caused by *H.pylori*, and as nitrite scavengers to prevent endogenous nitrosation leading to the carcinogenic N-nitroso compounds in the gastric lumen (Yamaguchi and Kakizoe, 2001).

High gastric cancer mortality in fugian province, China has been associated with consumption of certain salted fermented fish products. The major mutagen in these products is related to the nitrosation of methionine was demonstrated by Weisburger and colleagues (Chen, et al., 1995). China, with one of the world’s highest rate of gastric cancers has yielded clues to the environmental determinants of this tumour. A pilot study involving assays of urine and gastric juice of 60 individuals in a screening program showed higher levels of N-nitrosoproline and of cis- and *trans*-N-nitroso-2-methylthiazolidine-4-carboxylic acid among persons with gastric dysplasia than in normal controls or those with chronic atrophic gastritis (Chen et al., (1995). Sandhu et al., (Sandhu et al., 2001) reviewed 13 prospective cohort studies and stated that the risk of colorectal cancer was increased by 12 - 17%/100g of red meat consumed per day and by 49%/25g of processed meat consumed per day. Most of the processed meat was probably preserved with nitrite.

*N*-nitroso compounds undergo α-hydroxylation by CYP2E1 enzyme to form DNA adducts. The gene coding for this is polymorphic and thus may constitute a susceptibility factor for colorectal cancer. In a study conducted in Hawaii, it was found that subjects with one type of CYP2E1 variant were found to be at 60% increased risk (95% confidence interval, 1.1-2.5) for rectal cancer. The above subjects who were predicted to have been exposed levels of nitrosamines, based on their high intake of red meat or processed meat were at a markedly greater increased risk (2-fold
for red meat and 3-fold for processed meat) for rectal cancer (Marchand et al., 2002). Red meat that has been cooked to the degree termed 'well done' is a positive risk factor for colorectal cancer. Under high pyrolytic temperatures, heterocyclic amines and benzopyrene molecules can form inside and on surface of red meat. A major source of benzopyrene is red meat exposed to a naked flame, as occurs during the barbecuing process. In a study it was observed a 6% risk of large adenoma per 10ng/day consumption of BP. Consistent with this finding an incremental increase of 10g of barbecued red meat per day was associated with 29% increased risk of large adenoma (Gunter et al., 2005).

Endogenous N-nitrosation was specifically linked to ingestion of haem rather than inorganic iron or protein and may account for increased risk associated with red meat consumption in colorectal cancer. In this study, it was found that subjects fed with red meat have significantly greater levels of NOC with respect to those on low meat diet. They also observed that 8-mg supplement of haem iron also increased fecal NOC compared with low meat diet (Cross et al., 2003). In another study, the formation of N-nitroso compounds in upper GI tract was assessed in 27 ileostomists. These subjects were fed with diets containing no meat, or 240g red meat or 240g processed meat in a randomly cross over intervention design studies. The observations from the above studies indicated increased formation of N-nitroso compounds with red meat and processed meat consumption with respect to no meat diet. These studies have also observed that N-nitroso compounds were formed only in the presence of nitrosated haemoglobin, at pH 6.8 but not in the absence of nitrosated haemoglobin. These findings demonstrate that haem may facilitate the formation of NOC in the absence of colonic flora in the upper GI tract (Lunn et al., 2006).
The finding that high intake of red meat but not of chicken or fish might be associated with increased colon cancer risk was first reported in prospective studies of Willett et al. in 1990. The evidence of an inverse relationship between colon cancer risk and fish intake is less consistent than the evidence of a positive association with red meat. The European Prospective Investigation into Cancer and Nutrition (EPIC) studies on large western European population that includes half a million subjects from 10 European countries examined whether the associations exist between intakes of red and processed meat, of poultry, and of fish and colorectal cancer risk. EPIC studies have used a proportional hazards model adjusted for age, sex, energy (fat and non-fat sources) height, weight, work-related physical activity, smoking status, dietary fibre and folate, and alcohol consumption. They reported in this population study, that the absolute risk of development of colorectal cancer within ten years for a study subject aged 50 years was 1.73% for the highest category of red meat intake and 1.28% for the lowest category of intake and was 1.81% for subjects in the lowest category of fish intake and 1.28% for subjects in highest category of fish intake (Norat et al., 2005). The above results are further confirmed by randomised cross over design studies on human subjects fed with red meat, high red meat, high fibre and vegetarian diets. The exfoliated colonocytes recovered from human stool obtained from subjects on red and processed meat diet and vegetarian diet were measured for apparent total NOC. The level of NOC specific $O^6$-alkylguanine adducts, $O^6$MedG and $O^6$CMdG were significantly higher for the subjects on high red meat diet. Luminal NOC arising from red meat diet form alkylating DNA adducts in colonic cells, if not repaired, this may explain the association between red and processed meat and colorectal cancer (Lewin et al., 2006).
1.6 Nitrosation of Glycine and Other Amino acids - A Potential Source of Alkylating agents

Despite a continuous decline in the overall incidence, gastric cancer still remains a common cancer in both sexes. Many epidemiological studies unravelled a plethora of possible etiological factors responsible for gastric cancer. Our concern is mostly on protein-rich diet, exposure to nitrate, and nitrite precursors in dietary constituents.

Consumption of nitrite and dietary nitrogen containing compounds may result in the formation of nitrosamides and nitrosamines under the acidic conditions of the stomach. The latter compounds can form adducts with DNA and are carcinogenic leading to a potential risk for human gastrointestinal cancer.

Glycine, a simplest α-amino acid is among the most abundant amino acids in dietary proteins. Free glycine occurs in biological fluids at millimolar concentrations (Komorowska et al., 1981). It was reported in 1904 that nitrosation of glycine esters give rise to stable diazoacetic esters (Curtius, 1904). The formation of the salts of diazoacetic acid, were reported in 1908 (Muller, 1908). Apart from kinetics studies on decomposition, the potential toxicity and carcinogenicity of this simple compound have not been tested (Kreevoy and Konasewich, 1970). The resurgence of interest in chemistry and biology of diazo and N-nitrosopeptides was reviewed in 1989 (Challis, 1989). It was reported that diazo and N-nitrosopeptides are consistently mutagenic in many test systems and several are potent carcinogens in animal models (Anderson and Blowers, 1994).

Current models for analysis of total NOC do not permit identification of individual compounds. Hence there have been no studies to date directed at evaluating the possible contribution of these compounds to human
Figure 1.10: Mechanism of nitrosation of glycine and subsequent formation of $O^6$-MedG and $O^6$-CMdG. Nitrosation of glycine gives rise to alkylating agents which carboxymethylate and methylate DNA. Diazoacetic acid is formed as a common intermediate.

exposure. Further more DNA alkylation products of even simple nitrosated peptides are likely to be very complex. They are 20 different possible dipeptides for N-terminal glycine alone and the number of possible dipeptides of different amino acids increases arithmetically. Therefore, for the sheers reasons of practicality, in the first instance it was focussed on nitrosation of simple amino acids. Many studies reporting that nitrosoglycine derivatives react with nitric oxide to form diazonium ion, which reacts at nucleophilic $O^6$-position of 2-deoxyguanosine to give rise to pre-mutagenic adducts $O^6$-MedG and $O^6$-CMdG will be discussed in detail in next section [Figure 1.10] (Shuker, 2000).
1.6.1 $O^6$-carboxymethylguanine – A potential premutagenic lesion produced by nitrosoglycine derivatives

In several studies of DNA damage by nitrosated glycine derivatives the expected formation of carboxymethyl adducts $N^7$-carboxymethyl-2'-deoxyguanosine [7-CMdG], $N^5$-carboxymethyl-2'-deoxyguanosine [3-CMdG] and $O^6$-carboxymethyl-2'-deoxyguanosine [$O^6$-CMdG] was observed (Harrison et al., 1997; Shuker et al., 1987; Zurlo et al., 1982). Simple nitrosated bile acid conjugate N-nitrosoglycocholic acid reacts with DNA to give rise to $O^6$-CMdG and $O^6$-MedG. The latter has already been established as a toxic, promutagenic lesion and is repaired by DNA repair enzyme, $O^6$-alkylguanine-DNA-alkyltransferase (ATase). Calf thymus DNA treated with $^{14}$C-NOGC was hydrolysed by acid treatment and analysed by radio chromatographic HPLC. 7-CMdG, 3-CMdG and $O^6$-CMdG adducts were detected. The $^{14}$C labelled NOGC treated DNA was incubated with Atase from both prokaryotic and eukaryotic sources along with $^3$H –NMU labelled DNA. It was found that Atase got inactivated proportionally with the increasing concentrations of $^{14}$C-NOGC, which was determined by its inability to transfer methyl groups from $^3$H – MNU. This was determined by measuring radioactivity transferred to Atase by both scintillation counting and radio-chromatographic HPLC (Shuker and Margison, 1997).

When $^{14}$C-NOGC-treated calf thymus DNA hydrolysates were analysed by HPLC with fluorescence detection, a substantial peak of $O^6$-MedG was also detected along with $O^6$-CMdG. Interestingly $O^6$-CMdG alone was found, but not $O^6$-MedG when calf thymus DNA incubated with excess Atase. This suggests that $^{14}$C-NOGC treated calf-thymus DNA also formed $O^6$-MedG adduct, which under the conditions of incubation with Atase got completely repaired, but not $O^6$-CMdG. It was concluded from these observations that both methylation and carboxymethylation
Chapter 1: Introduction

Reaction of Nitroso-glycine derivatives with DNA to form Carboxymethyl diazonium ion

diazoacetic acid derivatives  N-nitroso-glycine derivatives

Potassium diazoacetate  APNG

Azaserine  N-Nitroso-glycocholic acid

Carboxymethyl diazonium ion

Figure 1.11: Various types of diazoacetic acid and N-nitroglycine derivatives. Both diazoacetic acid and N-nitroso-glycine derivatives give rise to a common reactive, potent alkylating intermediate, the carboxymethyl diazonium ion. APNG [N-(N'-acetyl-L-propyl)-N-nitroso-glycine], N-nitroso-glycocholic acid is a carcinogen and mutagenic derivative of the naturally occurring bile acid conjugate of glycocholic acid (Harrison, et al., 1999).
is a general property of nitrosoglycine derivatives suggesting $O^6$-CMdG is a useful biomarker of this pathway (Shuker and Margison, 1997).

Calf-thymus DNA has been treated with various nitrosated glycine derivatives such as potassium diazoacetate (KDA), N-acetyl-L-prolyl-$N$-nitrosoglycine (APNG), azaserine (AS) at varying concentrations and with a single concentration of mesyloxyacetic acid (MAA) to reconfirm the above results [Figure 1.11]. An assay for quantification of both $O^6$-CMdG and $O^6$-MedG adducts was developed, which involves affinity purification followed by HPLC/fluorescence analysis. The calf thymus DNA treated with nitroso-glycine derivatives was acid hydrolysed and both adducts are separated using immunoaffinity columns. The acid hydrolysates were directly analysed by HPLC. The results indicated that concomitant methylation and carboxymethylation is the general property of N-carboxymethyl-$N$-nitrosocompounds (APNG) as well as of diazoacetic acid derivatives. Different compounds yield different amounts of both adducts ruling out the possibility of single common intermediate such as carboxymethyl diazonium ion (Harrison et al., 1999).

The reported works described above have established that nitrosated glycine derivatives are potential DNA alkylating agents. Another interesting finding was that the major $O^6$-guanine adduct of nitrosated glycine derivatives, $O^6$-CMdG is not repaired by $O^6$-alkylguanine alkyl transferase and is likely to accumulate in the DNA of human gastro-intestinal tract. The formation of the characteristic $O^6$-carboxymethyl adducts by nitrosated glycine derivatives offers an approach to evaluate their overall contribution to the overall burden of diet related NOC exposures. For example 7-CMG is excreted unchanged in urine and could be used as a non-invasive marker (Harrison et al., 1999).
Previous *in vitro* studies with a range of nitrosated glycine derivatives had established that the ratio of $O^6$-CMdG to $O^6$-MedG ranged from 10:1 to 40:1 (Harrison et al., 1999). It has been suggested that such compounds are responsible for background levels of DNA methylation. The ratio of formation of $O^6$-CMdG to $O^6$-MedG appears to be 100:1 in human subjects and this would be consistent with the hypothesis that nitrosated glycine derivatives may be the source of damage (Shuker, 2000). Further support for this hypothesis comes from the volunteers, who are on protein intake, has demonstrated increased levels of total NOC (Bingham et al., 1996). A number of epidemiological studies have linked protein intake with increased risk of GI tract.

The role of dietary glycine in forming $O^6$-alkylguanine adducts at physiological relevant concentrations was further investigated. The amount of diazoacetate anion formed when glycine (10μM – 50mM) reacted with nitric oxide was observed using HPLC assay. The amount of the ion formed was measured using colorimetric measurement of nitrite. Diazoacetate anion formation depended linearly on glycine concentration. It was reported that at physiological concentrations of glycine and nitric oxide, the diazoacetate anion is not detectable. Solutions of nitrosated glycine reacted with 2′-deoxyguanosine and and calf-thymus DNA gave $O^6$-CMdG adducts, and at high concentrations of glycine and nitric oxide, $O^6$-MedG adducts were observed. Studies with synthetic diazoacetate anion indicated that concentrations < 14μM did not give detectable $O^6$-alkylguanine adducts. However $O^6$-CMdG adducts were detected in Human Blood samples obtained from three volunteers consuming a standardized high meat diet using Immunoslot Blot assay (Cupid et al., 2004).
Chapter 1: Introduction

1.7 Objectives of the Thesis

Glycine is a simple primary amino acid and is among the most abundant amino acids found in dietary proteins. Nitrosated glycine derivatives react with DNA to give rise to several adducts including $O^6$-carboxymethyl-2'-deoxyguanine adducts. Random mutagenesis studies on our routinely used laboratory model of $N$-nitrosoglycine derivative, potassium diazoacetate (KDA) using p53 yeast expression system indicated that this mutagen can induce transition mutations (C-T transitions) in the p53 Tumour suppressor gene via $O^6$-carboxymethyl-2'-deoxyguanine adducts (Gottschalg et al., 2006). These mutations were also observed in p53 gene obtained from the tissues of colon cancer patients. There is some indirect evidence that this is related to endogenous nitrosation of glycine. It was also previously reported that this adduct is not repaired by repair enzyme $O^6$-alkylguanine-DNA-alkyltransferase. In this context, it would be particularly interesting to study the physical, chemical and biological properties of $O^6$-carboxymethyl-2'-deoxyguanine adduct. The objectives of the studies reported in this project are outlined below.

- Synthesis and Characterisation of oligonucleotides containing $O^6$-alkylguanine adducts.
- Adduct site-specific mutagenesis studies on $O^6$-alkylguanine adducts using amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) assays.
- Carcinogen specific mutagenesis studies on $N$-nitrosoglycine derivative, potassium diazoacetate using HUPKI (Human p53 Knock-In) mouse embryonic fibroblast cell lines.
2.0 Materials and Methods

2.1 Materials

2.1.1 Reagents - Synthesis of O\textsuperscript{-}carboxymethylguanine phosphoramidite

The starting material 2'-deoxyguanosine [Catalogue No:05-0910-24] was obtained from CRUACHEM Ltd, Todd campus, West of Scotland Science Park Acre Road, Glasgow, G20, 0UA, Scotland.

2.1.2 Reagents Obtained from Acros Organics

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Catalogue No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic anhydride</td>
<td>14949 0010</td>
</tr>
<tr>
<td>N, N-dimethyl formamide</td>
<td>11622 0025</td>
</tr>
<tr>
<td>4-dimethylaminopyridine 99%</td>
<td>14827 0053</td>
</tr>
<tr>
<td>2-mesitylenesulfonyl chloride 99%</td>
<td>12559 0250</td>
</tr>
<tr>
<td>Methanol extra dry with molecular sieves</td>
<td>36439 1000</td>
</tr>
<tr>
<td>Phenylacetic acid 98%</td>
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</tr>
<tr>
<td>Pyridine extra dry with molecular sieves</td>
<td>36442 1000</td>
</tr>
<tr>
<td>Pyridine</td>
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<tr>
<td>Tetrahydrofuran</td>
<td>34845 1000</td>
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<tr>
<td>Triethylamine 99.5%</td>
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2.1.3 Reagents obtained from Sigma-Aldrich Company

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<tr>
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<th>Catalogue No</th>
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<tbody>
<tr>
<td>Acetonitrile Anhydrous 99.8%</td>
<td>27,100-4</td>
</tr>
<tr>
<td>Dichloromethane Anhydrous 99.8%</td>
<td>27,099-7</td>
</tr>
</tbody>
</table>
Chapter 2: Materials and Methods

2-cyanoethyl-N,N-diisopropylchloro phosphoramidite 30,230-9
Dichloromethane, anhydrous 99.8% 27,099-7
N,N-diisopropylethylamine 387649-100
1,8-diazabicyclo-(5.4.0)undec-7-ene 13,900-9
4,4-dimethoxytrityl chloride 95% 10,001-3
Ethyl diazoacetate E-22201
Methylglycolate 32,526-0

2.1.4 Laboratory Solvents obtained from Fischer Scientific
Acetonitrile, Chloroform, Dichloromethane, Diethyl ether, Ethyl acetate, Hexane, Methanol, n-pentane,

2.1.5 Reagents for DNA synthesis obtained from Glen Research products

2.15.1 Monomers used for DNA synthesis:
All the phosphoramidites used were ultra-mild β-cyanoethyl (CE) phosphoramidites

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Catalogue No</th>
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<tbody>
<tr>
<td>Pac-dA-CE phosphoramidite</td>
<td>10-1601-05E</td>
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<tr>
<td>Ac-dc-CE phosphoramidite</td>
<td>10-1015-05E</td>
</tr>
<tr>
<td>iPr-Pac-dG-CE Phosphoramidite</td>
<td>10-1621-05E</td>
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<tr>
<td>dT-CE Phosphoramidite</td>
<td>10-1030-05E</td>
</tr>
<tr>
<td>O(^6)-Me-dG-phosphoramidite</td>
<td>10-1070-02E</td>
</tr>
</tbody>
</table>

2.15.2 Solvents/Reagents used for DNA synthesis:

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Catalogue No</th>
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<tbody>
<tr>
<td>Activator</td>
<td>30-3102-52E</td>
</tr>
<tr>
<td>(0.45M Tetrazole sublimed in Acetonitrile)</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2: Materials and Methods

Anhydrous Wash 40-4050-53E
Diluent (acetonitrile anhydrous) 40-4050-50E
Cap Mix A 40-4212-52E
(9:1-THF/Acetic anhydride)
Cap Mix B 40-4212-52E
(0.02M 1-methylimidazole in THF/Pyridine/H2O)
Oxidising Solution 40-4132-52E
(0.02M I2 in THF/Pyridine/H2O)
Deblocking Mixture 40-4140-69E
(3% Trichloroacetic acid in Dichloromethane)

2.1.5.3 DNA supports Used for DNA synthesis:

<table>
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<tr>
<td>Pac-dA</td>
<td>20-2801-41</td>
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<tr>
<td>Ac-dC</td>
<td>20-2215-41</td>
</tr>
<tr>
<td>iPr-Pac-dG</td>
<td>20-2821-41</td>
</tr>
<tr>
<td>500A° CPG dT</td>
<td>20-2230-41</td>
</tr>
</tbody>
</table>

2.1.6 HPLC systems used for Characterization of Oligonucleotides

- Waters™ 2690 Separations Module equipped with Waters™ 996 Photodiode Array detector & Waters™ 474 scanning Florescence Detector.
- Waters™ 616 pump equipped with Waters™ 996 Photodiode Array detector & Waters™ 474 scanning Florescence Detector.
- Dionex BIOLC system equipped with a DIONEX variable wavelength detector.

2.1.6.1 HPLC and FPLC Columns Used:

- Waters™ X™erra® MS C18 (2.5µm, 4.6*50 mm) column on HPLC for purification analysis on longer oligonucleotides.
- Waters™ X™erra® MS C18 (3.5µm, 2.1*150 mm) column on HPLC for Fluorescence assays on base modified oligonucleotides.
- Pharmacia Mono Q HR 5/5 ion exchange column (Amersham Biosciences, Uppsala, Sweden, SE-75184) on FPLC for Preparative purification of Oligonucleotides.
- Atlantis (5µm, 4.5*25mm) C18 column on HPLC for nucleoside composition analysis on pentamers.
Chapter 2: Materials and Methods

- Phenomenex, Syngery 4μm, Hydro-RP 80A (2.00×150mm) column on HPLC for fluorescence assays on modified DNA to detect the presence of modified bases.

2.1.7 **Other Purification Kits used for DNA Purification:**

<table>
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<th>Company</th>
<th>Catalogue No</th>
</tr>
</thead>
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<tr>
<td>NENSORB™ PREP</td>
<td>Perkin Elmer Life Sciences</td>
<td>NLP-028</td>
</tr>
<tr>
<td>C18 Sep-Pak Reverse-Phase</td>
<td>Waters™ Corporation</td>
<td></td>
</tr>
<tr>
<td>Cartridges</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.1.8 **Enzymes used in this Research Project:**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Company</th>
<th>Catalogue No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>Sigma-Aldrich</td>
<td>P-5521</td>
</tr>
<tr>
<td>Nuclease P1</td>
<td>US Biological</td>
<td>N7000</td>
</tr>
<tr>
<td>Amplitaq Gold® DNA polymerase</td>
<td>Applied Biosystems</td>
<td>4311814</td>
</tr>
<tr>
<td>With 10X PCR Gold buffer &amp;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25mM of MgCl₂ Solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quick-Stick™ Ligase</td>
<td>BIOLINE</td>
<td>BIO-27027</td>
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</table>

Restriction enzymes used for Restriction Fragment analysis are obtained from New England Biolabs (UK) Ltd

Acl 1, Age 1, Apo 1, BbvC 1, Bmr 1, Bsa 1, Bst E 11, Bsu 36 1, Bts 1, Cla 1, Ear 1, EcoR 1, EcoR V Nae 1, Nar 1, Pci 1, Sac 1, Sca 1,

2.1.9 **Molecular Biology Kits and DNA ladders used in this Research Project:**

<table>
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<th>Product Name</th>
<th>Company</th>
<th>Catalogue No</th>
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<tr>
<td>QIAquick™ Gel purification Kit</td>
<td>QIAGEN</td>
<td>28704</td>
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<tr>
<td>QIAfilter™ Plasmid Midi Kit (25)</td>
<td>QIAGEN</td>
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<td>QIAEXII® Gel Extraction Kit</td>
<td>QIAGEN</td>
<td>20021</td>
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<tr>
<td>NOVABLUe Competent cells</td>
<td>Novagen</td>
<td>69825-4</td>
</tr>
<tr>
<td>Hyperladder 1V, quantitative</td>
<td>BIOLINE</td>
<td>BIO-33029</td>
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<tr>
<td>Tridye 1KB DNA Ladder</td>
<td>New England Biolabs (UK) Ltd</td>
<td>N3272S</td>
</tr>
</tbody>
</table>
Chapter 2: Materials and Methods

All the common laboratory chemicals used for adduct specific mutagenesis experiments including materials used for preparing gels such as Agaraose, polyacrylamide with $N,N$-methylenbisacrylamide, LB-Agar and LB-medium, Tris Base – 108g, Boric Acid – 55g, EDTA (pH 8.0) - 40μl, 10% APS and antibiotic penicillin were supplied by Dept of Pathology, University of Leeds, Leeds, UK.

All the common laboratory chemicals used for KDA mutagenesis studies such as Potassium hydroxide, chemicals used for cell culture such as DMEM Medium, 10% FCS, Trypsin, Trypan Blue dye and chemicals routinely used for DNA extraction such as 50mMTris-HCl, pH-8.0, 50mM EDTA, 1% Sodium dodecyl sulphate (SDS), 10mMNaCl, 20mg/ml proteinase K, Phenol, chloroform, Isopropanol, 70%ethanol, 3M sodium acetate pH - 5.2 were supplied by Dept of Genetic Alterations in Carcinogenesis, German Cancer Research Centre, Heidelberg, Germany.
Chapter 2: Materials and Methods

2.2 Methods Applied for Synthesis and Characterisation of Oligonucleotides Containing 2'-deoxyguanosine, O6- methyl-2'-deoxyguanosine and O6-carboxymethyl-2'-deoxyguanosine

2.2.1 Synthesis and Characterisation of 5'-O-(4,4-dimethoxytriphenylmethyl)-N2-phenylacetyl-O6-methoxycarbonylmethyl-2'-deoxyguanosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino)-phosphoramidite using NMR and LC-MS

The method, which was used to synthesise O6-CMdG is primarily based on the work done by Gaffney et al., (Gaffney and Jones, 1982). This is an unambiguous route involving the introduction of methyl glycolate at O6 position of 2'-deoxyguanosine by displacing an arylsulphonate group. In a modification developed by Xu (Xu, 2000), the 3' and 5'-OH groups were protected using acetyl groups instead of methoxyacetyl groups previously used by Harrison et al., (Harrison et al., 1997). The pivotal part of the synthesis, involving the introduction of methylglycolate moiety at O6 position of 2'-deoxyguanosine is similar in both methods. In the conventional synthesis, an isobutyryl group was used to protect exocyclic amino group. However, it has been documented that acyl groups protecting N2-position of O6-substituted 2'-deoxyguanosine were resistant to removal by ammonia than unsubstituted 2'-deoxyguanosine (Borow-Borowski and Chambers, 1987; Li and Swann, 1989; Smith et al., 1990). Therefore, phenylacetyl group, which is base labile, was used to protect the exocyclic 2-amino group of 2'-deoxyguanosine. This is because we cannot use ammonia for deprotection, since it converts ester at O6-position in to amide group. 0.4M triethylamine/methanol was used for deprotection of 3' and 5'-OH groups, because it is chemically specific and there is a less risk for removal of phenylacetyl group.
Compounds 1 - 7 were synthesised and characterised using NMR and LC-MS methods, according to reported procedure (Xu, 2000). Compounds 1-6 were characterised using proton ($^1$H) chemical shifts and compound 7 was characterised using $^{31}$P chemical shifts on NMR instrument with 300 MHz magnetic field from JOEL (JNM-LA 300) at the labs of chemistry, The Open University, Milton Keynes, UK. The chemical shifts were assigned by NMR technician Mr Gordon Howell and research assistant Mr. Praveen Patel at the Dept of Chemistry, The Open University, Milton Keynes, UK. 10mg of each compound was dissolved in DMSO-$d_6$ solvent to characterise $^1$H and $^{31}$P chemical shifts. The chemical shifts observed were in accordance with the Literature (Xu, 2000).

Compounds 1 – 6 were characterised on VG Micromass Quattro Triple Quadrupole LC-MS spectrometer. All the compounds were characterised by mass Spectrometry technician Mr Graham Jeffs, at the Dept of Chemistry, The Open University, Milton Keynes, UK. The eluant system used for characterisation was 50/50 acetonitrile/0.02 NH$_4$AC (pH 5.4). Since, the samples were injected directly from the autosampler therefore no column was used. Compounds were 1 – 6 were characterised using positive ion mode LC-MS. The recorded m/z data was in agreement with the literature (Xu, 2000).
2.2.1.1 Synthesis of 3', 5'-diacetyl-2'-deoxyguanosine (compound 1)

6 g (21 mmol) of 2'-deoxyguanosine was dissolved in 68 ml pyridine and 108 ml of DMF in a round bottomed flask. Acetic anhydride (12.5 ml) was added slowly drop by drop to the reaction mixture at room temperature [Figure 2.1]. The reaction was monitored for 48 hrs using TLC (chloroform and methanol (10/90) solvent system) (Li and Swann, 1989).

Since this reaction takes a long time to go for completion, DMAP was added as a catalyst to increase the rate of the reaction at the room temperature. The reaction was finished in just 2-3 hrs. Pyridine was removed under vacuum and the product was recrystallised from 95% ethanol (7.4 g) Yield: 93%. UV $\lambda_{\text{max}}$ 254 nm $^1$H NMR data (in DMSO-d$_6$): 2.04 (3H, s, acetyl at 5'), 2.09 (3H, s, acetyl at 3'), 2.44-2.98 (2H, m, 2'-H and 2''-H), 4.17-4.32 (3H, m, 4'-H and 5'-H), 5.33 (1H, d, 3'-H), 6.15 (1H, dd, 1'-H), 6.56 (2H, s, 2-NH$_2$), 7.90 (1H, s, 8'-H) and 10.66 (1H, s, N-H). MS m/z: [2MH$^+$] found at 702.12, (MH$^+$) found at 351.32, (MH$^+$ - Sugar fragment) found at 151.77.

![Figure 2.1: The protection of 3' and 5' hydroxyl groups of sugar moiety by acetyl groups using acetic anhydride reagent.](image)
2.2.1.2 Protection of Exocyclic 2'-Amino group by Phenylacetylation (compound 2)

Phenylacetylation of the exocyclic amino group was carried out using phenylacetyl chloride. Comparatively better yields of phenylacetylated product can be achieved with phenylacetic anhydride than chloride. Phenylacetyl chloride is highly reactive forming many side products, which makes separation difficult and gives low yields. However, phenylacetic anhydride is unstable and the protection was done using phenylacetyl chloride. The advantage of using phenylacetyl moiety for protecting exocyclic amino group is that it remains stable during deprotection of acetyl groups by triethylamine in methanol.

2.2.1.2.1 Synthesis of Phenylacetic anhydride

Phenylacetic anhydride was prepared by the reaction between phenylacetyl chloride and sodium salt of phenylacetic acid. Phenylacetic acid (13.6g, 0.1M) was added to a stirred aqueous solution of sodium hydroxide (0.1M in 15ml) to prepare sodium salt of phenyl acetic acid. 5 drops of pyridine was added followed by slow addition of phenylacetyl chloride (15.46 g, 0.1M).

Reactions:

\[
\text{C}_6\text{H}_5\text{CH}_2\text{COOH} + \text{NaOH} \rightarrow \text{C}_6\text{H}_5\text{CH}_2\text{COONa} + \text{H}_2\text{O}
\]

\[
\text{C}_6\text{H}_5\text{CH}_2\text{COONa} + \text{C}_6\text{H}_5\text{CH}_2\text{COCl} \rightarrow (\text{C}_6\text{H}_5\text{CO})_2\text{O} + \text{NaCl}
\]

Finally, the reaction mixture was washed with water to remove NaCl. Since, NaCl is an inorganic salt it can be easily removed by washing with water. However, phenylacetic anhydride is highly unstable and hydrolyses on contact with water. Hence, similar reaction was repeated using total non-aqueous phase. Sodium salt of
phenylacetic acid was dissolved in ethyl acetate solvent to which phenylacetyl chloride was added. After the completion of reaction, the desired product was precipitated in ether solvent. This procedure does not give acceptable yields.

2.2.1.2.2 Protection of Exocyclic 2'-Amino group of 2'-deoxyguanosine with Phenylacetic anhydride

5g (14.2nmol) of 3',5'-diacetyl-2'-deoxyguanosine was dried by evaporation of a pyridine solution (30ml). Phenylacetic anhydride (20g, 85mmol) in pyridine (70ml) was then added and the solution stirred (120° C, 30min) (Li and Swann, 1989). Pyridine was evaporated and the reaction mixture was dissolved in 100ml of chloroform and washed with 100ml of sodium bicarbonate thrice. The organic layer was dried (MgSO₄) and is evaporated under reduced pressure to a thick gum. The gummy residue was applied to silica gel and desired product was purified by methanol/chloroform (10/90) solvent system.

![Figure 2.2: The protection of exocyclic 2'-amino group of 2'-deoxyguanosine sugar moiety by phenylacetyl group using phenylacetyl chloride reagent.](image)

Figure 2.2: The protection of exocyclic 2'-amino group of 2'-deoxyguanosine sugar moiety by phenylacetyl group using phenylacetyl chloride reagent.
2.2.1.2.3 Protection of Exocyclic 2'-Amino group of 2'-deoxyguanosine using Phenylacetyl chloride

3', 5'- diacetyl-2'-deoxyguanosine (4g, 11.3mmol) was dissolved in pyridine (30ml) and evaporated. Pyridine (50ml) was added and the reaction was stirred at room temperature. Phenylacetyl chloride is highly reactive and induces exothermic reaction when reacted with amine. Hence, the reaction system was kept cooled by keeping it stirring in an ice-bath. Phenylacetyl chloride (10ml, 55.36mmol) was added slowly drop by drop. The reaction was monitored using TLC with methanol/chloroform (5/95) solvent system. The reaction mixture was left overnight and additional amount of phenylacetyl chloride (1-2ml) was added to complete the reaction [Figure 2.2]. Pyridine was evaporated and the reaction mixture was dissolved in 100ml of chloroform and washed with 100ml of sodium bicarbonate thrice. The organic layer was dried (MgSO₄) and is evaporated under reduced pressure to a thick gum. This gummy residue was applied to silica gel and desired product was purified by methanol/chloroform (10/90) solvent system. The desired product containing fractions were pooled together, evaporated and dried to form yellowish powder (3g) Yield: 46%. UV \( \lambda_{\text{max}} \) 259nm \(^1\text{H} \) NMR data (in DMSO-d₆): 2.02 (3H, s, acetyl at 5''), 2.08 (3H, s, acetyl at 3''), 2.54 -2.96 (2H, m, 2'-H and 2''-H), 3.80 (2H, s, CH₂ of phenylacetyl), 4.14-4.47 ( 3H m, 4'-H and 5'-H), 5.31 (1H, d, 3'-H), 6.22 ( 1H, dd, 1'-H), 7.23-7.34 (5H, m, of C₆H₅), 8.24 (1H, s, 8'-H), and 11.94 ( 1H, s, N₂-H, ex). MS m/z: (MH⁺) found at 470.40, (MH⁺ - sugar fragment) found at 269.56 [MH⁺ - Sugar Fragment - PAC] – 151.53
2.2.1.3 Mesitylenesulphonylation of $O^6$-position of $N^2$-phenylacetyl-3',5'-diacetyl-2'-deoxyguanosine (compound 3)

3',5'-diacetyl-$N^2$-phenylacetyl-2'-deoxyguanosine (3g, 6.4mmol) was dissolved in DCM (100ml), to which TEA (3ml), DMAP (30mg) and mesitylenesulphonyl chloride (1.5g, 6.8mmol) were added sequentially. Reaction was monitored using TLC with methanol/chloroform (10/90) solvent system. After 30 min about two-thirds of the starting material ($R_t$-0.5) was converted into a new spot with $R_t$ - 0.8. Then additional amount of mesitylenesulphonyl chloride (0.5g) was added and the solution left stirred for another two hours to complete the reaction [Figure 2.3]. The solvent was evaporated under the vacuum and applied to a silica gel column and eluted with chloroform. The fractions containing the product were pooled together and concentrated to a small volume for recrystallisation. However, this compound was found to be quite unstable and gave poor yields on isolation. Hence, it was not isolated by column during subsequent experiments and used without purification in the subsequent step (Xu, 2000).

![Figure 2.3: Addition of mesitylenesulphonyl group at $O^6$ position of 2'-deoxyguanosine using mesitylenesulphonyl chloride reagent.](image-url)
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The crystals formed were washed with diethyl ether, collected by filtration, and dried to give crystals of 3.9g (yield: 93%). UV $\lambda_{\text{max}} = 279\text{nm}$. $^1\text{H}$ NMR data (in DMSO-d$_6$): 1.98 (3H, s, acetyl at 5'), 2.20 (3H, s, CH$_3$ at 4-position of mesitylenesulphonyl), 2.06 (3H, s, acetyl at 3'), 2.54 (6H, s, 2×CH$_3$ at 2 and 6 positions of mesitylenesulphonyl), 2.89-2.98 (2H, m, 2'-H and 2''- H) 3.82 (2H, s, CH$_2$ of phenylacetyl), 4.24-4.36 (3H, m, 4'-H and 5'-H), 5.40 (1H, d, 3'-H), 6.30 (1H, t, 1'-H), 6.38 (2H, s, 2' and 5' positions of mesitylenesulphonyl) 7.19-7.36 (5H, m, 5H of phenylacetyl) 8.02 (1H, s, 8'-H) and 10.78 (1H, s, N2-H ex)
2.2.1.4 Synthesis of 3',5'-diacetyl-\(N^2\)-phenylacetyl-\(O^6\)-methoxycarbonylmethyl-2'-deoxyguanosine (compound 4)

3',5'-diacetyl-\(N^2\)-phenylacetyl-\(O^6\)-mesitylenesulphonyl-2'-deoxyguanosine (2.9g, 4.46mmol) was dissolved in dried acetonitrile (20ml), to which quinuclidine (0.77g, 6 mmol) was added. The solution was kept stirred at room temperature. After 30 Minutes TLC (10/90, methanol/chloroform) had showed that all of the starting material is converted in to a new spot with its \(R_t\) being near zero. Then methylglycolate (2 ml, 25mmol) and 1, 8-diazabicyclo (5,4,0) undec-7-ene (DBU) (0.6 ml, 3.9 mmol) were added sequentially. After 2 hrs, about quarter of the starting material was converted in to a new spot with \(R_t\) around 0.8. Then additional amount of methylglycolate (0.5ml) and DBU (0.3ml) were added and the solution was left stirred overnight [Figure 2.4].

![Figure 2.4: Substitution of \(O^6\)-position of 2'-deoxyguanosine with methoxycarbonylmethyl group by displacing mesitylenesulphonyl group with quinuclidine followed by methylglycolate.](image)

After the completion of reaction, solvent was evaporated under the vacuum and the residue was dissolved in ethyl acetate (100ml) and washed with saturated aqueous
solution of sodium chloride thrice (3×100ml). The organic layer was dried over sodium sulphate, concentrated and applied to silica gel to elute with chloroform, collected and dried to give compound 4 (in foam, 1.9g, yield: 75%). UV $\lambda_{\text{max}}$ = 267nm. $^1$H NMR data (in DMSO-d$_6$): 2.00 (3H, s, acetyl at 5'), 2.06 (3H, s, acetyl at 3'), 2.47-2.95 (2H, m, 2'-H and 2''-H), 3.64 (3H, s, OCH$_3$), 3.94 (2H, s, CH$_2$ of phenylacetyl), 4.25-4.41 (3H, m, 4'-H and 5'-H), 4.98 (2H, s, CH$_2$ of O$_6$-CH$_2$), 5.37 (1H, m, 3'-H), 6.35 (1H, t, 1'-H), 7.19-7.33 (5H, m, C$_6$H$_5$), 7.92 (1H, s, 8'-H), 10.68 (1H, s, N2-H, ex). MS m/z: (MH$^+$) found at 542.44, (MH$^+-$sugar fragment) found at 342.24, (MH$^+$-sugar fragment-Pac group) found at 224.03. C$_{25}$H$_{29}$O$_9$ N$_5$ requires 542.1887.
2.2.1.5 Synthesis of $N^2$-phenylacetyl-\(O^6\)-methoxycarbonylmethyl-2'-deoxyguanosine (compound 5)

3',5'-diacetyl-$N^2$-phenylacetyl-\(O^6\)-methoxycarbonylmethyl-2'-deoxyguanosine (1.7g, 3.15mmol) was dissolved in 25 ml of 0.4M triethylamine in methanol. Deprotection of protecting groups was monitored using TLC (10/90) methanol/ chloroform solvent system. The acetyl groups of the starting material were removed gradually to from three new spots. Spots with $R_t$ - 0.7 and $R_t$ - 0.5 represent 3' and 5'- deacetylated products and spot with $R_t$ - 0.35 represents $N^2$-phenylacetyl-\(O^6\)-methoxycarbonylmethyl-2'-deoxyguanosine (5). The reaction mixture was left stirred overnight to let the reaction go for completion to form desired compound $N^2$-phenylacetyl-\(O^6\)-methoxycarbonylmethyl-2'-deoxyguanosine (5) with $R_t$ - 0.35. Solvent was evaporated under the vacuum and the solid residue left was co­-evaporated with absolute alcohol once and toluene twice to give white foam (compound 5) [Figure 2.5].

\[
\begin{align*}
\text{(4)} & \\
\text{(5)}
\end{align*}
\]

Figure 2.5: The deprotection of 3' and 5' hydroxyl groups of 2'-deoxyguanosine using triethylamine in methanol reagent.

The residue was recrystallized from methanol to give white crystals of compound 5 about 950mg. Yield: 62.5%. UV $\lambda_{max} = 269$nm. $^1$H NMR data (in DMSO-d$_6$): 2.23-
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2.74 (2H, m, 2'-H and 2''-H), 3.57 (2H, m, 5'-H), 3.64 (3H, s, OCH₃), 3.79 (2H, s, CH₂ of Phenylacetyl), 3.84 (1H, m, 4'-H), 4.40 (1H, m, 3'-H), 4.89 (1H, t, 5'-OH), 5.17, (2H, s, CH₂ of O₆-CH₂), 5.30 (1H, d, 3'-OH), 6.32 (1H, t, 1'-H), 7.19-7.34 (5H, m, C₆H₅), 8.48 (1H, s, 8'-H), 10.64 (1H, s, N₂-H). MS m/z: (MH⁺) found at 458.30, (MH⁺-sugar fragment) found at 342.61, (MH⁺-sugar fragment-Pac group) found at 224.05. C₂₁H₂₄O₇N₅ requires 458.1676.
2.2.1.6 Synthesis of 5'-O-(4,4'-dimethoxytriphenylmethyl)-N²-phenylacetyl-O⁵-methoxycarbonylmethyl-2'-deoxyguanosine (compound 6)

*N²*-phenylacetyl-O⁵-methoxycarbonylmethyl-2'-deoxyguanosine (450mg, 0.98M) was dissolved in anhydrous pyridine (10ml) and the solution was cooled in an ice bath. DMAP (10mg) and DMT-Cl (340mg, 0.001M) were added sequentially. Reaction was monitored on TLC using ethyl ether and then (10/90) methanol / chloroform solvent system. After completion of the reaction, pyridine was evaporated under the vacuum and the concentrated residue was applied to silica gel to purify the compound with methanol/chloroform (10/90) solvent system. Since, DMT group is sensitive to the acidic nature of silica, the basic conditions on the column were maintained by adding a few drops of pyridine to eluting solvent. The fractions were pooled together, evaporated and the resulting residue was precipitated from ice-cold pentane [Figure 2.6].

Figure 2.6: The addition of 4',4'-dimethoxytriphenylmethyl (DMT) group on 5'-OH position of 2'-deoxyguanosine is essential for inter-nucleotide chain formation during automated DNA synthesis.

The precipitate was the dried to give a slightly yellowish powder (450mg). Yield: 65%. ¹H NMR data (in DMSO-d₆): 2.30-2.85 (2H, m, 2'-H and 2"-H), 3.07 (2H,m, 5'-H and 5"-H), 3.65 ( 3H, s, OCH₃), 3.69 (6H, s, 4,4-OCH₃ of DMT), 3.77 (2H, s, CH₂
of phenylacetyl), 3.94 (1H, m, 4'-H), 4.45 (1M, m, 3'-H), 5.18 (2H, s, CH$_2$ of O$_6$-CH$_2$), 5.30 (1H, d, 3'-OH), 6.35 (1H, t, 1'-H), 7.13-7.32 (18H, m, 5H of phenylacetyl and 13H of DMT), 8.36 (1H, s, 8'-H), 10.60 (N2-H, ex). MS m/z: (MH$^+$) found at 759.54, (MH$^+$ - DMT) found at 302.24.
2.2.1.7 Synthesis of 5'-O-(4,4'-dimethoxytriphenylmethyl)-N²-phenylacetyl-O⁶-methoxycarbonylmethyl-2'-deoxyguanosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino)-phosphoramidite (compound 7)

DNA with compound 7

DNA with O⁶-CMdG Phosphoramidite

Figure 2.7: The addition of 3'-O-(2-cyanoethyl-N,N-diisopropylamino)-phosphoramidite at 3'-OH position of 2'-deoxyguanosine is essential for inter-nucleotide chain formation during automated DNA synthesis. Compound 7 is directly incorporated into an oligonucleotide sequence and deprotected with 0.5M NaOH solution to obtain final sequence with O⁶-CMdG adduct.

5'-O-(4,4'-dimethoxytriphenylmethyl)-N²-phenylacetyl-O⁶-methoxycarbonylmethyl-2'-deoxyguanosine was dissolved in dried THF (5ml) and diisopropylethylamine (0.6ml) was added to the reaction mixture. Under constant stirring 0.2ml of 2'-
cyanoethyl-\(N,N\)-diisopropylchloro phosphoramidite was added. The mixture was stirred for half an hour at room temperature by which period starting material was converted to two higher \(R_t\) products as checked by TLC (dichloromethane/ethylacetate/diisopropylethylamine) in the ratio 75:25:2. Both the products are stereo isomers of the compound 7. The mixture was diluted with 30ml of ethyl acetate and washed with saturated NaHCO\(_3\) (3×30ml). The organic phase was dried over sodium sulphate and evaporated to a small volume, then co-evaporated with toluene to an oily residue. The residue was purified by chromatography on silica gel eluting both the products with dichloromethane/ethylacetate/diisopropylethylamine in the ratio (75:25:2). The fractions containing both the desired products were combined, evaporated and precipitated with ice-cold pentane and freeze dried with benzene to give a white powder (120mg) [Figure 2.7]. Compound 7 is the precursor of \(O^6\)-carboxymethyl-2'-deoxyguanosine phosphoramidite. Initially the compound 7 was directly incorporated in to the oligonucleotide sequence. Then final sequence containing compound 7 was deprotected with 0.5M NaOH to remove functional protecting groups to form \(O^6\)-carboxymethyl-2'-deoxyguanosine phosphoramidite. Yield: 46%. \(^{31}\)P NMR data (in DMSO-d\(_6\)): 151.15 and 151.20
2.2.2 Synthesis of Oligonucleotides containing Normal 2'-deoxyguanosine, O⁶-methyl-2'-deoxyguanosine and O⁶-carboxymethyl-2'-deoxyguanosine using Expedite™ 8908 instruments

Merrifield (Merrifield, 1963) and Letsinger (Letsinger et al., 1964) originally developed the concept of solid phase synthesis simultaneously for peptide synthesis. Later this idea is adapted for oligonucleotide synthesis by Letsinger (Letsinger and Mahadevan, 1966). This concept has four basic aspects, which are as follows

1. The oligonucleotide is synthesised while attached covalently to a solid support.

2. Excess soluble protected nucleotides and coupling reagent can drive a reaction near to completion.

3. The reaction is carried out in a single reaction vessel to diminish mechanical losses due to solid support manipulation, allowing synthesis with minute quantities of starting materials.

4. Heterogeneous reactions are standardised, and these procedures are easily automated.

β-cyanoethyl phosphoramidite chemistry is the method of choice in DNA synthesis. This method provides the following advantages.

1. High coupling efficiency

2. Reduced side reactions, which maintain high biological activity

Each DNA synthesis cycle is composed of four steps with intervening wash steps to remove excess of reagents and reaction by products.

The steps involved in each synthesis cycle are as follows

1. Deblocking

2. Coupling

3. Capping
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4. Oxidation

5. Capping

Following the final capping step, all the steps are repeated again, until chain elongation is completed (Xu, 2002).

1. **Deblocking:** The monomers, which will be used on Expedite systems for DNA synthesis, consists of 5-OH protecting group, DMT (4,4'-dimethoxytrityl group) on the sugar moiety. This step involves cleavage of 5'-OH protecting dimethoxytrityl group, which can be confirmed by observing orange colour liquid flowing through trityl waste. The intensity of the colour indicates the quality of the reaction, which is essential for good coupling yield.

2. **Coupling:** This step involves coupling of incoming phosphoramidite monomer to the monomer on the solid support. They cannot simply couple to each other. This involves activation by tetrazole (proton abstractor), a weak acid. Free 5'-OH acts as a nucleophile by donating proton to the dialkyl amino group, forming a reactive intermediate.

3. **Capping:** The coupling reaction is not quantitative in a finite period. A small percentage of truncated sequences will always be produced at the end. If these truncated sequences are allowed to react further, it will be difficult to isolate product from sequence mixture. This problem is overcome by acetylating free OH groups. Acetic anhydride is used as a capping reagent to acetylate free OH groups, thereby preventing failure sequences to react further.

4. **Oxidation:** The newly formed phosphite internucleotide linkage is highly unstable and susceptible to both acidic and basic conditions. This trivalent phosphite group is oxidised to pentavalent phosphate group. Iodine is used as mild
oxidant in basic tetrahydrofuran solution with water as the oxygen donor. This reaction is extremely fast.

Oligonucleotides Sequences Synthesised for Physical and Biochemical studies

(a) Nucleoside Composition Analysis:
1. 5' AGGCT 3' (Standard Pentamer)
2. 5' AGXCT 3' (X = O^6-methylguanine)
3. 5' AGYCT 3' (Y = O^6-carboxymethylguanine)

(b) Sequences for Melting point \((t_m)\) Analysis:
4. 5' CGC GAG CTT GCG 3' \((t_m - \text{Standard Sequence})\)
5. 5' CGC GAG CTC GCG 3' \((t_m - \text{Standard Sequence})\)
6. 5' CGC XAG CTT GCG 3' \((X = O^6-\text{Methylguanine})\)
7. 5' CGC XAG CTC GCG 3' \((X = O^6-\text{Methylguanine})\)
8. 5' CGC YAG CTT GCG 3' \((Y = O^6-\text{Carboxymethylguanine})\)
9. 5' CGC YAG CTC GCG 3' \((Y = O^6-\text{Carboxymethylguanine})\)

(c) Sequences used for Mutagenesis Studies:
10. 5' TCA GCA TCT TAT CCG AGT GGA AGG A 3' (Standard 25mer)
11. 5' AAT TTC CTT CCA CTC GGA TAA GAT GC 3' (Standard 26mer)
12. 5' TCA GCA TCT TAT CCX AGT GGA AGG A 3' \((X = O^6-\text{Methylguanine})\)
13. 5' AAT TTC CTT CCA CTC XGA TAA GAT GC 3' \((X = O^6-\text{Methylguanine})\)
14. 5' TCA GCA TCT TAT CCY AGT GGA AGG A 3' \((Y = O^6-\text{Carboxymethylguanine})\)
15. 5' AAT TTC CTT CCA CTC YGA TAA GAT GC 3' \((Y = O^6-\text{Carboxymethylguanine})\)

Table 2.1: Sequences of modified oligonucleotides synthesised on Expedite™ 8909 series instruments DNA Synthesiser.

The final capping involves capping free OH groups with acetic anhydride. If the amino group of guanine is protected with phenoxyacetyl group, then phenoxyacetic
anhydride should be used as a capping agent to avoid partial replacement with acetic anhydride (Xu, 2002).

Oligonucleotides of different lengths were synthesised to serve different purposes. Pentamers were mainly synthesised because these are short sequences, which makes it easy to detect $O^6$-alkylguanine adducts incorporated in to these sequences. 12mer is an ideal length for any self-complementary sequence to study the melting temperature of DNA adducts. Despite a large number of characterised G-mutations specific to potassium diazoacetate (KDA) (Gottschalg et al., 2006), none of these sites between codons 120-260 of the p53 sequence lay within a short sequence between two unique restriction sites which lies between codons 192-200. Codon 196 was the site chosen for insertion of modified DNA adduct. 25 and 26mers were chosen because they are complementary p53 sequences occurring between two unique restriction sites and have codon 196 at which $O^6$-alkylguanine adducts could be incorporated. These complementary sequences can form duplex containing single $O^6$-alkylguanine adduct, which will allow construction of an adducted shuttle vector for mutagenesis studies. CPG support was removed from the column, dissolved in 1ml of ammonium hydroxide for normal oligonucleotides, and left overnight at room temperature. Next day the oligonucleotide in ammonium hydroxide solution was left at 55°C for two hours. Ammonium hydroxide cleaves the oligonucleotide from the solid support and removes other protecting groups as well. The modified oligonucleotide containing $O^6$-methyl -2'-deoxyguanosine adduct were deprotected by dissolving the CPG support in 1ml of 10% DBU in methanol solution and left in dark for 5 days. The oligonucleotides containing $O^6$-methoxycarbonylmethyl-2'-deoxyguanosine adduct were deprotected by dissolving solid support in 1ml of 0.5M aqueous sodium hydroxide solution overnight.
Note: The mode of oligonucleotide synthesis which was used in this work is DMT-ON i.e., 5-DMT (4,4'-dimethoxytrityl chloride) of the final base at 5'-end will not be cleaved. This group plays an important role during separation of full length sequences from truncated sequences.

All the oligonucleotides were synthesised at the scale of 1 μmole. The sequences of oligonucleotides synthesised were given in Table 2.1. The monomers and supports were obtained from Glen research, Virginia, 20164, USA. The synthesis was carried out on Expedite™ 8909 series instruments [PE Biosystems, Foster city, CA 94404] DNA synthesizer.

2.2.3 Purification of Oligonucleotides using NENSORB™ PREP cartridges

Milligram quantities of Trityl-on oligonucleotides were purified and detritylated using this cartridges. The oligonucleotides in 0.1M triethylammonium acetate (TEAA) buffer pH 7.0 or concentrated ammonium hydroxide were applied to the NENSORB™ PREP cartridges. Salts, failure sequences and synthetic by-products were washed away while the DMT-ON oligonucleotides remain bound to the cartridge. The trityl group was then hydrolysed from the 5'-end of the oligonucleotide with a 0.5% trifluoroacetic acid wash, leaving the trityl-off oligonucleotide bound to the cartridge. Subsequent recovery of the purified, trityl-off oligonucleotide was accomplished with a 35% methanol wash.

Procedure:

1. Activate the cartridge with 10ml of methanol.

2. Pre-equilibrate the cartridge with 5ml of 0.1M TEAA buffer (pH 7.0).
3. Add the oligonucleotide in the concentrated ammonium hydroxide to the cartridge and pull the sample in to the resin bed until the meniscus reaches the top of the bed.

4. Wash the cartridge with 10 ml of (1:9) acetonitrile / 0.1M TEAA (pH 7.0) solution. Failure sequences, salts, and synthetic by-products will be washed away during this procedure, while DMT-ON oligo remains bound to the column.

5. Wash the cartridge with 25 ml of 0.5% TFA. The trityl group is hydrolysed in this step and trityl-off oligo remains bound to the support.

6. Wash the support with 10 ml of 0.1M TEAA, pH 7.0, to remove any traces of acid left on cartridge.

7. Prepare 10ml of (35%) methanol solution. Elute the support using 10ml of this solvent system and collect ten fractions of 1ml each.

8. Check the OD\textsubscript{260} value of each fraction (1:10 dilutions are usually required). Oligo is usually found between fraction 3 to fraction 5.

9. Dry the desired fractions using lyophilisation or vacuum centrifugation

Oligonucleotides concentration is referred in the terms of OD units. One OD unit is defined as the amount of product, which has an absorbance of 1.0 when dissolved in 1ml buffer (i.e., 20mM sodium phosphate, 0.1M NaCl, pH 7.0), and measured in 1cm cuvette at 20° c at absorbance 260nm.
2.2.4 Preparative Purification of Oligonucleotides using Fast Protein Liquid Chromatography

The synthesis of oligonucleotides containing normal bases has become routine, but recently interest in the preparation of modified oligonucleotides has increased, due to overwhelming evidence implicating the role for $O^6$-alkylguanine and $O^4$-alkylthymine in mutation and carcinogenicity. In many molecular biological experiments, the effects due to modified oligonucleotides are obscured by the presence of impurities of even small amount of oligonucleotides, especially its parent oligo containing normal bases. Therefore, it is of great importance to produce modified oligomers with high purity.

At present short oligomers are purified by preparative PAGE using neutral buffer. The recovery of DNA using this process is not quantitative. This might be due to technical difficulties in extraction process. Reverse Phase HPLC is also used to separate short oligomers using weakly acidic buffer (e.g., KH$_2$PO$_4$ buffer, pH 6.3). Though it is very effective in separating short oligomers differing by one nucleotide, reverse phase HPLC is not good at separating oligomers of medium length i.e. 24mers. When modified oligomers are prepared, the problem is to separate the oligomer containing the base of interest from oligomers of same length, which can be difficult to achieve.

These modified oligomers are in particular useful for point mutation studies. Xu and Swann have optimised a method to separate and purify oligomers of identical length but different base composition (Xu and Swann, 1992). This is called FPLC (Fast protein liquid chromatography). This technique is based on separation by charge difference between oligomers. Certain anion exchange columns can be used at high pH 12. Under these basic conditions not only phosphates (pK$_a$ 6.8)
display negative charges but also imino protons of thymine and guanine can dissociate and display negative charge. Therefore, oligonucleotides of identical length, but different base composition should be separable by an-ion exchange chromatography at higher pH.

Liquid chromatography was carried out on a Dionex BIOLC system with a DIONEX variable wavelength detector using Mono Q HR 5/5 column [Amersham Biosciences, Uppsala, Sweden, SE-75184] equipped with UV detector. The gradient is given in the Table 2.2

<table>
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<th>Time [min]</th>
<th>Flow [ml/min]</th>
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<th>% Buffer B</th>
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**Table 2.2: FPLC gradient used for Preparative Purification of Oligonucleotides**

**Buffer A**: 0.4M NaCl in 10mM NaOH (pH 11)
Dissolve sodium chloride (23.376g) in 750ml of water and add 10ml of 1M aqueous sodium hydroxide to the above solution and make up to 1ltr with sterile water.

**Buffer B**: 1.2M NaCl in 10mM NaOH (pH 11)
Dissolve sodium chloride (70.128gms) in 750ml of water and add 10ml of 1M aqueous sodium hydroxide to the above solution and make up to 1ltr with sterile water.
The maximum pressure and flow rate recommended for this column were 580 psi and 3 ml/min. Hence, buffers were used at a flow rate of 1 ml/min at optimal pressure 360 psi. The amount of sample injected on HPLC for analysis and preparative purification purposes were 10 and 200 μl. The percentage of the buffer B was increased gradually to 100% to elute the desired component. Chromatography was monitored at 260 nm.

The oligonucleotides eluted using the above buffers will not remain stable due to high pH and presence of salts. Hence, the fractions containing oligos were neutralised with 5 μl of 50% acetic acid solution. The fractions of oligos were desalted using C18 Sep-Pak Reverse-phase cartridge.

2.2.5 Desalting of Oligonucleotides Using C18 Sep-Pak Reverse-Phase Cartridges

The procedure was as follows

1. Prepare a C18 Sep-Pak(TM) cartridge by washing with 15 - 20 ml of HPLC-grade acetonitrile.
2. Wash the column with 10 ml of distilled or sterile water.
3. Add the FPLC fractions containing the oligonucleotide to the column.
4. Wash the column with 10 ml of distilled or sterile water to remove salt.
5. Elute the column with 5 ml of 50% acetonitrile solution. Collect five fractions of 1 ml each in an eppendorf tube.

The OD260 values of each fraction were measured on UVICON photo spectrometer from BIOTEK instruments.
2.2.6 FPLC Analysis of Pentamers containing $O^6$-alkylguanine adducts: $O^6$-methyl-2'-deoxyguanosine and $O^6$-carboxymethyl-2'-deoxyguanosine

Pentamer analysis was carried out on Dionex BIOLC system with a DIONEX variable wavelength detector using Mono Q HR 5/5 column [Amersham Biosciences, Uppsala, Sweden, SE-75184] equipped with UV detector. The gradient is given in the Table 2.3.

<table>
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<th>Flow [ml/min]</th>
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<th>% Buffer B</th>
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</tr>
<tr>
<td>30.0</td>
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</tr>
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</table>

Table 2.3: FPLC gradient used for the characterisation of pentamers.

**Buffer A:** 0.0M NaCl in 10mM NaOH (pH 11)

Prepare the above solution by adding 10ml of 1M aqueous sodium hydroxide to 1ltr of distilled water.

**Buffer B:** 0.4M NaCl in 10mM NaOH (pH 11)

Dissolve sodium chloride (23.376gms) in 750ml of water and add 10ml of 1M aqueous sodium hydroxide to the above solution and make up to 1ltr with sterile water.

The maximum pressure and flow rate recommended for this column were 580psi and 3ml/min. Hence, buffers were used at a flow rate of 1ml/min at optimal pressure 360psi. The percentage of buffer B was rapidly increased to 40%
by 5 minutes and then gradually to 100% by 25 minutes to determine the retention time of each individual pentamer. Chromatography was monitored at 260nm.

2.2.7 HPLC Analysis of Oligonucleotides Containing Normal 2'-deoxyguanosine, O6-methyl-2'-deoxyguanosine and O6-carboxymethyl-2'-deoxyguanosine

HPLC analysis of O6-alkylguanines containing oligonucleotides was carried out on Waters™ 2690 Separations Module using XTerra R MS C18 (2.5μm, 4.6×50 mm) column equipped with Waters™ 996 Photodiode Array detector. Analysis was monitored at 260 nm at a flow rate of 1ml/min and gradient is given in the Table 2.4.

<table>
<thead>
<tr>
<th>Time</th>
<th>%A</th>
<th>% B</th>
<th>% C</th>
<th>Column ° C</th>
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<td>60° C</td>
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<td>60° C</td>
</tr>
<tr>
<td>22.05</td>
<td>80</td>
<td>20</td>
<td>0</td>
<td>60° C</td>
</tr>
</tbody>
</table>

Table 2.4: Gradient used for characterisation of the purity of the longer oligomers (25 and 26 mers).

Solvents Used:

**Mobile Phase A:** 5% Acetonitrile in 0.1M TEAA Buffer

**Mobile Phase B:** 15% Acetonitrile in 0.1M TEAA Buffer

**Mobile Phase C:** 100% Acetonitrile
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2.2.8 Nucleoside Composition Analysis

1.0 OD units of standard pentamers AGGCT, AGXCT and AGYCT were dissolved in 160μl of water and 10μl of 0.6M Tris-HCl, 0.06M MgCl₂ (pH-4.5) solution. 10 units of Nuclease P1 was added to the reaction mixture and incubated for 2 hrs (AGGCT, AGXGCT) and overnight for AGYCT at 37°C. 20μl of 0.6M Tris-HCl, 0.06M MgCl₂ (pH-8.0) buffer was added followed by 10 units of alkaline phosphatase and incubated for 2hrs in a water bath at 37°C. This reaction was followed on Reverse Phase HPLC using Atlantis (5μm, 4.5×25mm) C₁₈ column on Waters™ 616 pump equipped with Waters™ 996 photodiode Array detector. 0.01M KH₂PO₄ buffer (pH-6.52)/Acetonitrile was used as a mobile phase, monitoring at 260nm at a flow rate of 1ml/min. The gradient is given the Table 2.5.

<table>
<thead>
<tr>
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<th>%C</th>
<th>%D</th>
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</tr>
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</table>

Table 2.5: Gradient used for HPLC characterization of modified pentamers using Nucleoside Composition Analysis.
Solvents Used:

**Mobile Phase A:** Methanol

**Mobile Phase B:** Water

**Mobile Phase C:** Acetonitrile

**Mobile Phase D:** 0.01M KH$_2$PO$_4$ buffer (pH-6.52)

### 2.2.9 Fluorescence Analysis of 25 and 26mers containing Modified Guanine bases: $O^6$-methyl-$2'$-deoxyguanosine and $O^6$-carboxymethyl-$2'$-deoxyguanosine.

1.0 OD units of 25 and 26mers containing modified $2'$-deoxyguanosine bases ($O^6$-methyl-$2'$-deoxyguanosine and $O^6$-carboxymethyl-$2'$-deoxyguanosine) were dissolved in 160μl of water and 10μl of 0.6M Tris-HCl, 0.06M MgCl$_2$ (pH-4.5) solution. 10 units of Nuclease P1 was added to the reaction mixture and incubated 2 hrs for the oligonucleotides containing $O^6$-methyl-$2'$-deoxyguanosine and overnight for the oligonucleotides containing $O^6$-carboxymethyl-$2'$-deoxyguanosine at 37°C. 20μl of 0.6M Tris-HCl, 0.06M MgCl$_2$ (pH-8.0) buffer was added followed by 10 units of alkaline phosphatase and incubated for 2hrs in a water bath at 37°C. This reaction was followed on Reverse Phase HPLC using Phenomenex, Synergy 4μm, Hydro-RP 80A (150×2.00mm) column on Waters™ 2690 Separations Module equipped with Waters™ 996 Photodiode Array detector and Waters™ 474 scanning fluorescence detector. 0.01M KH$_2$PO$_4$ buffer (pH-6.52)/Acetonitrile was used as a mobile phase, monitoring at excitation wavelength 286nm and emission at 378nm at a rate of 0.25ml/min. The gradient is given the Table 2.5.
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<table>
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<th>Time</th>
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<th>%C</th>
<th>%D</th>
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<td>75.0</td>
<td>5.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

**Table 2.6:** HPLC gradient used for characterization of oligonucleotides (mutation assay sequences 12-15) containing O6-alkylguanine adducts using fluorescence detector.

**Solvents Used:**

*Mobile Phase A:* Methanol

*Mobile Phase B:* Water

*Mobile Phase C:* Acetonitrile

*Mobile Phase D:* 0.01M KH2PO4 pH-6.62

2.2.10 Melting curve (Tm) Measurements on Modified Self –Complementary DNA Sequences.

Melting curve measurements were performed in stoppered quartz cuvettes using UVICON XL spectrophotometer from BIOTEK instruments, which is connected to a programmable thermo system (Peltier system) from BIOTEK instruments. This
programmable thermo system is capable of maintaining the temperature in cell holder over a range from 10°C - 100°C in a 10mm path length, 500μl capacity quartz cell. The self-complementary DNA strand was dissolved in 0.1M Hepes buffer at pH -7.0. The temperature dependent change in absorbance was measured at 260nm. The rate of temperature increase was 1°C/min. The T_m values were determined as the maximum values of the first derivative graph of the absorbance vs. temperature.
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2.3 Methods applied for Characterisation of Mutations Induced by O\textsuperscript{6}-alkylguanine adducts Incorporated Site-specifically in to p53 cDNA Sequence

2.3.1 Extraction of Plasmid pLS76 DNA using QIAfilter Plasmid Midi kit

Plasmid, pLS76 is a 9kb p53 expression vector, was first described and constructed by Ishioka (Ishioka et al., 1993) [Figure 4.3]. It expresses full length p53 cDNA from the ADH1 constitutive promoter [Appendix 3]. The vector also contains CYCI (terminator) downstream of the p53 cDNA, yeast origin of replication, CEN6/ARS4 and LEU2 as a selectable marker. Plasmid, pLS76 DNA was used to construct a shuttle vector containing single O\textsuperscript{6}-carboxymethyl-2'-deoxyguanosine adduct for our mutagenesis studies. Plasmid DNA was extracted from E.coli using QIAfilter plasmid midi purification kit from QIAGEN ltd. This kit can be used to purify up to 100\(\mu\)g of low or high copy number plasmid or cosmid DNA. The advantage of QIAfilter cartridges over conventional centrifugation is it enables filtration to clear bacterial lysates.

A single colony was picked up and streaked on to a fresh LB-agar plate containing 100\(\mu\)g/ml of ampicillin. 2ml of starter culture (LB medium) containing ampicillin (100\(\mu\)g/ml) was inoculated with single E.coli colony containing plasmid, pLS76. Starter culture was incubated for ~8hrs at 37° C with vigorous shaking (~200 rpm) in an orbital shaker. Then this starter culture was diluted at a ratio of 1part per 50 parts in to LB medium containing ampicillin 100\(\mu\)g/ml and incubated at 37° C for 12 – 16 h with vigorous shaking (~200 rpm). The bacterial cells were harvested by centrifugation at ~4000 rpm for 15min at 4° C. The pellet was resuspended in 4ml of buffer P1 (50mM Tris-Cl, pH 8.0; 10mM EDTA, 100\(\mu\)g/ml RNase A). (Note: RNase A should be added to buffer P1 to get a final concentration
of 100μg/ml before using). The bacterial cells were completely re-suspended by vortexing or pipeting up and down until no cell clumps remain. 4ml of buffer P2 (200mM NaOH, 1% (w/v) SDS) was added to allow lysis of bacterial cells in suspension. Buffer was mixed gently by inverting 4-6 times and incubated at room temperature for 5min. **(Note: Do not vortex and do not allow lysis reaction to proceed no more than 5 minutes).**

During the incubation, QIAfilter cartridge was prepared by screwing the cap onto the outlet nozzle. QIAfilter cartridge is a device, which is similar to a syringe with a screw top outlet. This was used at later stage to filter bacterial lysate. 4ml of chilled buffer P3 (3.0M potassium acetate, pH5.5) was added to neutralise the lysate. The lysate was mixed by shaking 4-6 times immediately, but gently. The buffers were mixed thoroughly to allow neutralisation proceed to completion. On adding buffer P3, a fluffy white precipitate containing genomic DNA, proteins, cell debris and SDS becomes visible. This lysate was immediately transferred in to the barrel of the QIAfilter cartridge and incubated for 10 min. **(Note: Do not insert plunger in to the barrel).** Meanwhile a QIAGEN-tip is equilibrated using 10ml of Buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol (v/v), 0.15% Triton X-100 (v/v). QIAGEN-tip 100 is a portable chromatographic column from which we can elute the desired components by changing the pH of the eluate. After 10 min of incubation, outlet nozzle of QIAfilter cartridge was unscrewed. The plunger was inserted gently and cell lysate was filtered in to previously equilibrated QIAgen-tip 100. **(Note: Do not apply extreme force).**

The cleared lysate containing plasmid DNA and other contaminants enters the QIAGEN-tip by gravity flow and binds to the resin. QIAGEN-tip was washed with 2* 10ml of buffer QC (1.0M NaCl, 50mM MOPS (pH
7.0), 15% isopropanol (v/v). First wash is sufficient to remove contaminants in all major plasmid preparations. Second wash is necessary if bacterial strains producing large amounts of carbohydrates are used. This was followed by extraction of plasmid DNA by eluting the QIAGEN-tip with 5ml of buffer QF (1.25M NaCl, 50mM Tris-Cl (pH 8.5), 15% isopropanol (v/v). Buffer QF fraction containing plasmid DNA was precipitated by adding 3.5ml of room-temperature isopropanol. The precipitate was mixed and centrifuged immediately at ~4000 rpm for 30min at 4° C. Isopropanol pellets have a glassy appearance and are more difficult to see than salt containing pellets that result from ethanol precipitation. Hence, due care was taken during decanting the supernatant. These pellets were washed with 2ml of room-temperature 70% ethanol and centrifuged at ~4000 rpm for 10min. The supernatant was carefully decanted with out disturbing the pellet. The pellet was air-dried for 5-10 min and re-dissolved in 300μl of TE buffer (pH 8.0).

2.3.2 Construction of Genetically Engineered Plasmid, pLS76 containing O\(^6\)-alkylguanine Adducts

2.3.2.1 Double digestion of Plasmid, pLS76 DNA with \textit{BbvC} and \textit{SacI} Restriction enzymes

30μg of plasmid DNA was added to a clean 1.5ml micro-centrifuge tube. 10μl of both 10X NE buffer 4 and 10μl of 10X BSA were added to the plasmid DNA and mixed thoroughly by pipeting up and down. Five units of restriction enzymes \textit{BbvC} \(I\) and \textit{SacI} \(I\) were added to the above reaction mixture and mixed thoroughly by brief centrifugation. The reaction mixture was incubated overnight at 37° C in a water bath.
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2.3.2.2 Double digestion of Plasmid, pLS76 DNA with ApoI and SacI Restriction enzymes

Enzymes Apo I and Sac I differ in their reaction temperatures as well as in their percentage of activities in different NE buffers. Both these enzymes were showing considerable amount of activity in NE Buffer 3. Hence, NE buffer 3 was chosen for this double digestion reaction. Apo I requires 50° C to react, where as Sac I reacts at 37° C. Therefore, it was decided to cut the plasmid DNA with both enzymes one after another. 30µg of plasmid DNA was added to a clean 1.5ml micro-centrifuge tube. 10µl of both 10X NE buffer 3 and 10µl of 10X BSA were added to the plasmid DNA mixed thoroughly by pipeting. Initially five units of Apo I, was added to the reaction mixture and incubated at 50° C for 6-8 h. Later, five units of SacI was added to the above reaction mixture and incubated overnight at 37° C in a water bath.

2.3.2.3 Extraction of BbvC I + Sac I and Apo I + Sac I Fragments Using QIAquick Gel Extraction Kit from QIAGEN.

This is a protocol based on using microcentrifuge and is designed to extract and purify DNA fragments of 70bp to 10kb from standard and low-melt agarose gels in TAE or TBE buffer. Both the BbvC I + Sac I and Apo I + Sac I fragments were extracted from the respective excised DNA fragments from the agarose gel using a clean and sharp scalpel. These gel slices were weighed in a colourless centrifuge tube (approximately 200-400mg). Three volumes of buffer QG (Composition of this buffer is not mentioned anywhere by Qiagen. However, it contains chaotropic salt, guanidine thiocyanate which acts as a pH indicator) was added for 1 volume of gel (100mg ~ 100µl). The gel slices in buffer QG were dissolved by incubating at 50° C for 10 min and dissolving was aided by vortexing the sample tubes every 2-3 min during
incubation. The mixture was checked for its yellow colour (similar to buffer QG without dissolved agarose) which indicates that pH of the buffer QG is 7.5. Any change in pH is clearly indicated by change in the colour of the mixture to either violet or orange. This is due to the presence of the pH indicator in Buffer QG. This change in the pH of buffer QG will affect the DNA adsorption to Qiaquick membrane, which was used in the later stage. One gel volume of isopropanol was added to each agarose gel slice (100mg ~ 100μl) and mixed thoroughly.

A QIAquick spin column was placed in a provided 2ml collection tube. This is a device similar to micro-centrifuge tube with a filter to collect elute. The sample in buffer QG was applied to this QIAquick column and centrifuged for 1 min at 13,000 rpm in a bench top centrifuge. (Note: The maximum volume of the column reservoir is 800μl). The flow-through was discarded and the QIAquick column is placed back in to the same collection tube again. Another 0.5ml of buffer QG was added to the QIAquick column and centrifuged for 1 min at 13,000 rpm to remove any additional traces of agarose. 0.75ml of buffer PE was added to wash the QIAquick column and centrifuged for 1 min at 13,000 rpm. (Note: add 96%–100% ethanol to buffer PE before use). The flow-through was discarded and the QIAquick column is centrifuged again at 13,000 rpm for 1 min to remove any residual ethanol. This QIAquick spin column was transferred to a clean 1.5ml eppendorf tube. The DNA fragments were eluted by adding 50μl of buffer EB (10mM Tris-Cl, pH 8.5) to the centre of the QIAquick membrane and centrifuging the column at 13,000 rpm for 1 min. Purity of the DNA fragments extracted from agarose gel were checked on 1% agarose gel.
Table 2.7: Different types of duplexes constructed with the adduct present either on sense or anti-sense strand.

- Duplex 1: Normal 25mer (sequence 10) and Normal 26mer (sequence 11) were annealed to form control DNA duplex 1.
- Duplex 2: Modified 25mer containing O6-MedG adduct (sequence12) and Normal 26mer (sequence 11) were annealed to form DNA duplex 2 with O6-MedG adduct on sense strand.
- Duplex 3: Normal 25mer (sequence 10) and Modified 26mer containing O6-MedG (sequence 13) were annealed to form DNA duplex 3 with O6-MedG adduct on Anti-sense strand.
- Duplex 4: Modified 25mer containing O6-CMdG adduct (sequence 14) and normal 26mer (sequence 11) were annealed to form DNA duplex 4 with O6-CMdG adduct on sense strand.
- Duplex 5: Normal 25mer (sequence 10) and Modified 26mer containing O6-CMdG adduct (sequence 15) were annealed to form DNA duplex 5 with O6-CMdG adduct on Anti-sense strand.

2.3.2.4 Annealing of Complementary Single stranded 25mer and 26mers to Construct Single O6-alkylguanine Adduct Containing duplex DNA.

Despite a large number of characterised G-mutations specific to potassium diazoacetate (KDA)(Gottschalg et al., 2006), none of these sites between codons 120-260 of the p53 sequence lay within a short sequence between two unique restriction sites which lies between codons 192-200. Codon 196 was the site chosen for insertion of modified DNA adduct at nucleotide position 587 on sense strand and at nucleotide position 586 on anti-sense strand. The sequences chosen for mutagenesis studies are complementary sequences (sequences numbered 10-15) [see Table 2.1 and Figure 4.8]. Dr. Phil Burns, Dept of Pathology, University of Leeds designed these
sequences. Sequences 10 and 11 are standard 25 and 26mers chosen from tumour suppressor gene, p53 cDNA comprising codon 190 to codon 202. The sequences 12 and 13 are methylated counterparts and sequences 14 and 15 are carboxymethylated counterparts of standard sequences. Annealing reactions were carried out on a PCR heat block, PTC-200 Peltier thermal cycler, DNA engine from MJ Research. 2.5nmol (20µg) of each single stranded oligonucleotide (25mer and 26mer) were mixed in a microcentrifuge tube and heated up to 95° C on a PCR heat block to denature the DNA fragments. Then it was rapidly cooled down to 70° C. It was left for 2 minutes at 70° C to prevent any chances of forming secondary structures. The reaction mixture was cooled down slowly to room temperature in an hour. The complementary sequences were annealed in such a way that duplex formed will contain adduct either on sense or anti-sense strand. The information on types of duplex constructed is given in Table 2.7.

2.3.2.5 Separation of Annealed duplex DNA from Single Stranded Oligonucleotides using 19:1 Non-Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

2.3.2.5.1 Preparation of 19:1 Polyacrylamide Gel:

**10X TBE Buffer:**

- Tris Base – 108g
- Boric Acid – 55g
- EDTA (pH 8.0) - 40µl

10% APS: Take 1g of Ammonium persulphate and dissolve it in 10ml of distilled water.
8ml of 40% w/v 4x crystallised polyacrylamide with $N, N$-methylenebisacrylamide $5C \pm 0.2$ in deionised water solution was mixed with 4ml of 10X TBE buffer. The volume was made up to 20ml with distilled water. 450μl of 10% APS and 45μl of TEMED were added at the same time. The solution was swirled to mix completely and poured between the glass plates fixed with spacers to set the gel.

Acrylamide is a monomer, which in the presence of free radicals supplied by ammonium persulfate and stabilised by TEMED, a chain reaction is initiated in which monomers of acrylamide are polymerised in to long chains. When the bifunctional agent $N, N$ – methylenebisacrylamide is included in the polymerisation reaction, the chain becomes cross-linked to form a gel. Annealed oligonucleotides were loaded on to gels with 6x type1 loading buffer (0.25% Bromophenol blue, 0.25% Xylene Cyanol and 40% w/v sucrose in H$_2$O) to separate from single stranded oligonucleotides in 1X TBE buffer.

2.3.2.5.2 Isolation of Duplex DNA Fragments from Polyacrylamide Gel Using User Developed Protocol from QIAGEN

This procedure has been adapted from QIAquick Gel extraction kit protocol and Sambrook et al., (Sambrook et al., (1989). Polyacrylamide gel was stained in ethidium bromide solution and the band of interest was located by visualising the gel under UV light. The band of interest was excised using clean sharp scalpel and transferred to a clean microfuge tube. The gel slice was weighed in a microfuge tube and crushed against the wall of the tube. 1-2 volumes of diffusion buffer (0.5M ammonium acetate; 10mM magnesium acetate; 1mM EDTA, pH 8.0; 0.1% SDS) was added to 1 volume of gel (100mg ~ 100-200μl). The samples were incubated at 37° C overnight in a water bath. The samples were centrifuged for 1 min and the supernatant was
carefully removed using a pipette and transferred into a clean microfuge tube. An additional 0.5 volume of diffusion buffer was added to the gel slice and centrifuged for 1 min. The two supernatants were combined by mixing. The supernatant was filtered using a GF/C disposable sterile filter to remove any traces of polyacrylamide gel. The volume of the supernatant was determined and 3 volumes of buffer QG was added to 1 volume of gel (100mg ~ 300μl). A Qiaquick spin column was arranged in a 2ml collection tube. The supernatant in buffer QG was applied to QIAquick spin column to bind DNA and centrifuged for 1 min. The flow-through was discarded and the spin column was placed in the same collection tube. The column was washed by adding 0.75ml of buffer PE and centrifuged for 1 min. The flow-through was discarded and Qiaquick column was placed back in the same collection tube. The column was centrifuged for another 1 min to remove any residual traces of ethanol. Qiaquick column was transferred into a clean microfuge tube. 50μl of elution buffer (10mM Tris-Cl, pH 8.5) was added to the centre of Qiaquick column to elute DNA fragments.

2.3.2.6 Ligation of BbvCl + SacI and ApoI + SacI and Annealed Duplex DNA using Quick-Stick™ Ligase Kit to form an Intact Plasmid with Single O6-alkylguanine adduct.

Unfortunately, cohesive ends formed by BbvCl restriction enzyme were not very sticky. Quality control assay of this enzyme claims after 2-fold over digestion with BbvCl < 5% of the DNA fragments can be religated with BbvCl. Hence, ligation reactions were tried at 2-fold, 4-fold and 8-fold concentrations of BbvCl + SacI double stranded DNA. 8-fold reactions were repeated multiple times to get enough circularised plasmid for transformations in to E.coli cells. Five different types of
plasmids were constructed of which one is control plasmid, two of them were plasmids containing methylated adduct present either on sense strand or anti-sense strand and the remaining two were carboxymethylated plasmids with adduct present either on sense strand or anti-sense strand [Table 2.7]. The reaction was assembled in a microfuge tube by mixing all the three DNA fragments in the appropriate ratios given in the Table 2.8. 1μl of the enzyme, QS ligase was added to the reaction mixture followed by addition of 5μl of 4X QS buffer. Contents were mixed thoroughly by pipetting. The reaction mixture was incubated at room temperature for 5 minutes. The ligation mixture was analysed by running 5μl of the sample on 1% agarose gel.

**Reaction Contents:**

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</tr>
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Table 2.8: 2,4 and 8 fold amounts of BbvC1 + SacI double stranded DNA were used to find an optimal concentration of double stranded plasmid DNA required to construct an intact adduct containing plasmid.
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Types of Plasmids Constructed:

- Control plasmid — Plasmid with duplex 1
- Plasmid, pLS76 - 2 — Plasmid with duplex 2
- Plasmid, pLS76 - 3 — Plasmid with duplex 3
- Plasmid, pLS76 - 4 — Plasmid with duplex 4
- Plasmid, pLS76 - 5 — Plasmid with duplex 5

- Duplex 1: Normal 25mer (sequence 10) and Normal 26mer (sequence 11) were annealed to form control DNA duplex 1.
- Duplex 2: Modified 25mer containing $O^6$-MedG adduct (sequence 12) and Normal 26mer (sequence 11) were annealed to form DNA duplex 2 with $O^6$-MedG adduct on sense strand.
- Duplex 3: Normal 25mer (sequence 10) and Modified 26mer containing $O^6$-MedG (sequence 13) were annealed to form DNA duplex 3 with $O^6$-MedG adduct on Anti-sense strand.
- Duplex 4: Modified 25mer containing $O^6$-CMdG adduct (sequence 14) and normal 26mer (sequence 11) were annealed to form DNA duplex 4 with $O^6$-CMdG adduct on sense strand.
- Duplex 5: Normal 25mer (sequence 10) and Modified 26mer containing $O^6$-CMdG adduct (sequence 15) were annealed to form DNA duplex 5 with $O^6$-CMdG adduct on Anti-sense strand.

Table 2.9: Different types of plasmids, pLS76 containing O6-alkylguanine adducts constructed

2.3.3 Transformations of Ligation Mixture in to NOVABLU E. coli

Competent cells using Standard kits

Novablue competent cells (Merck biosciences) enable convenient, efficient construction of plasmid recombinants. They can be obtained both as standard and single kits. The cells in the standard kits were provided as frozen 0.2ml aliquots and each vial can be used for 10 transformations. The cells in the single kits were provided as single-use-50μl aliquots for greater efficiency and convenience, and were packed in kits for either 11 or 22 transformations. Appropriate numbers of competent cell aliquots were taken including one extra sample for the test plasmid positive control. All the tubes were immediately placed in the ice, so that all but the cap is
immersed. The cells were allowed to thaw on ice for 2-5 minutes. They were visually checked to see that they have thawed by gently flicking the tubes 1-2 times to evenly resuspend the cells. The cells were then immediately aliquoted. Ten 1.5ml microfuge tubes were pre-chilled by keeping them on ice. 20μl aliquots of cells were pipetted into the pre-chilled tubes. 1μl (0.2ng) of test plasmid was added to one of the tubes containing cells to determine transformation efficiency. 1μl of (1/10) and (1/100) dilutions of ligation mixture and purified plasmid were added to the separate tubes containing competent cells. Cells were stirred gently to mix and returned to ice, so that the tube was remained immersed in the ice, except for the cap. The tubes were incubated on the ice for 5 minutes. Heat shock was given to the cells by placing them in a water bath at 42° C for 30 sec (do not shake). The tubes were placed on ice for 2 minutes. 80μl of room temperature SOC (Full form of SOC abbreviation was not mentioned anywhere in the literature) medium was added to each tube. (Do not remove cells from ice until all have received SOC). The tubes were incubated at 37° C by shaking at 250rpm for 30 minutes prior to plating them on selective media. During outgrowth, the selective plates containing ampicillin (50μg/μl) were dried by placing them in an incubator at 37° C. (Note: If the plates contain a lot of moisture, place them cover-side up and open the cover 1/3 of the way to allow the plates to dry for 30 – 45 min). The plates were removed from the incubator and 5μl of transformants containing test plasmid, 5μl and 50μl volumes of transformants containing ligation mixture and purified plasmid were transferred on to selective plates by spreading them with bent glass rod. (If plating less than 25μl, it is best plating it on a pool of SOC). The plates are incubated overnight at 37° C.
2.3.4 Amplification of p53 cDNA from Plasmid, pLS76 Containing 2'-deoxyguanosine and O6-methyl-2'-deoxyguanosine and O6-carboxymethyl-2'-deoxyguanosine Adducts by Polymerase Chain Reaction

An appropriate volume (2μl) of DNA template (plasmid pLS76 DNA containing either normal 2'-deoxyguanosine or O6-alkylguanine adducts) was mixed with 5μl of 10X PCR gold buffer (Mg$^{2+}$ Free) (Applied Biosystems, Foster City, USA), 2μl of 25mM MgCl$_2$ solution (Applied Biosystems, Foster City, USA), 4μl of 2.5mM dNTP mix, 1μl of primer BB5 (5'-Biotin-CCAGGTCCAGATGAAGCTCC-3', 10pmol/μl), 1μl of primer NBE3 (5'-GGAGAGGAGCTGGTGTTGTT-3', 10pmol/μl), 0.25μl AmpliTag plus DNA polymerase (5U/μl) (Catalogue no-4311814, Applied Biosystems, Foster City, USA) and water to make up final volume to 50μl. The DNA was amplified using the following program [95° C/9 min, (95° C/1min, 55° C/1min, 72° C/2min) * 30times) 72° C/5min, and hold at 4° C for an hour] in the MJ Research, DNA Engine, PTC-225 (programmable thermal controller) PCR machine.

2.3.5 Detection of Mutations in Adducted Plasmid, pLS76 using ARMS-PCR (amplification refractory mutation system)

p53 cDNA was amplified from both normal plasmid and adducted plasmid constructs. An appropriate volume (2μl) of DNA template was mixed with 5μl of 10X PCR gold buffer (Mg$^{2+}$ Free) (Applied Biosystems, Foster City, CA, USA), 2μl of 20mM MgCl$_2$ solution (Applied Biosystems, Foster City, CA, USA), 4μl of 25mM dNTP mix, 0.25μl of AmpliTag plus DNA polymerase (5u/μl) (Catalogue no-4311814,
Chapter 2: Materials and Methods

Applied Biosystems, Foster City, USA) and site-specific primer sequences depending on the reaction.

**Sequences of sequence specific primers used for ARMS-PCR assay with Plasmid, pLS76-2 and plasmid, pLS76-4:**

**Sequence specific primers for analysis on Anti-sense strand which differ at 3’-end**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>3’-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer AG</td>
<td>5’ CCC TCC TCA GCA TCT TAT CCG</td>
<td>3</td>
</tr>
<tr>
<td>Primer AC</td>
<td>5’ CCC TCC TCA GCA TCT TAT CCC</td>
<td>3</td>
</tr>
<tr>
<td>Primer AA</td>
<td>5’ CCC TCC TCA GCA TCT TAT CCA</td>
<td>3</td>
</tr>
<tr>
<td>Primer AT</td>
<td>5’ CCC TCC TCA GCA TCT TAT CCT</td>
<td>3</td>
</tr>
</tbody>
</table>

**Conserved primers:**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>3’-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer SI</td>
<td>5’ TGT GAT GAT GGT GAG GAT GG</td>
<td>3’</td>
</tr>
<tr>
<td>Primer S2</td>
<td>5’ CTT CCA GTG TGA TGG TGA</td>
<td>3’</td>
</tr>
</tbody>
</table>

**Sequence specific primers for analysis on Sense strand which differ at 3’-end**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>3’-end</th>
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</thead>
<tbody>
<tr>
<td>Primer SC</td>
<td>5’ ACG CAA ATT TCC TTC CAC TC</td>
<td>3’</td>
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<tr>
<td>Primer SG</td>
<td>5’ ACG CAA ATT TCC TTC CAC TG</td>
<td>3’</td>
</tr>
<tr>
<td>Primer SA</td>
<td>5’ ACG CAA ATT TCC TTC CAC TA</td>
<td>3’</td>
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<tr>
<td>Primer ST</td>
<td>5’ ACG CAA ATT TCC TTC CAC TT</td>
<td>3’</td>
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**Conserved primers:**

<table>
<thead>
<tr>
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<th>Sequence</th>
<th>3’-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer A1</td>
<td>5’ CAA CTG GCC AAG ACC TGC</td>
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<tr>
<td>Primer A2</td>
<td>5’ CCC TCA ACA AGA TGT TTT GCC</td>
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Table 2.10: Sequence specific primers used for base pairing analysis on plasmid, pLS76 - 2 containing methylated adduct and plasmid, pLS76 - 4 containing carboxymethylated adduct on sense strand at nucleotide position 587 in codon 196. Primers used for analysis on Sense strand were given prefix (S) and for Anti-sense strand (A) will differ at 3’end, which is indicated in red colour. Conserved primers for sense strand were prefix (S) and for anti-sense strand (A).

In the Chapter 4 Prefixes will be not used in the explanation of ARMS-PCR assay and conserved primer sequences were given just for information purpose. These will not be mentioned in the chapter 4.

**Sequences of sequence specific primers used for ARMS-PCR assay with Plasmid, pLS76-3 and plasmid, pLS76-5**

**Sequence specific primers for analysis on Anti-sense strand which differ at 3’-end**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Primer AC</td>
<td>5’ CCC CTC CTC AGC ATC TTA TCC</td>
<td>3’</td>
</tr>
<tr>
<td>Primer AA</td>
<td>5’ CCC CTC CTC AGC ATC TTA TCA</td>
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</tr>
<tr>
<td>Primer AG</td>
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</tr>
<tr>
<td>Primer AT</td>
<td>5’ CCC CTC CTC AGC ATC TTA TCT</td>
<td>3’</td>
</tr>
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</table>
Conserved primers:
Primer S1  5’ CAA ACA CGC ACC TCA AAG C 3’
Primer S2  5’ CGC ACC TCA AAG CTG TTC C 3’

Sequence specific primers for analysis on Sense strand which differ at 3’-end
Primer SG  5’ ACG CAA ATT TCC TTC CAC TCG 3’
Primer SA  5’ ACG CAA ATT TCC TTC CAC TCA 3’
Primer SC  5’ ACG CAA ATT TCC TTC CAC TCC 3’
Primer ST  5’ ACG CAA ATT TCC TTC CAC TCT 3’

Conserved Primers:
Primer A1  5’ TCT GTG ACT TGC ACG TAC TC 3’
Primer A2  5’ ATT CTG GGA CAG CCA AGT CT 3’
Primer A3  5’ AGT CTG TGA CTT GCA CGT ACT C 3’
Primer A4  5’ GTC TGT GTG CAC GTA CT 3’

Table 2.11: Sequence specific primers used for base pairing analysis on plasmid, pLS76 - 3 containing methylated adduct and plasmid, pLS76 - 5 containing carboxymethylated adduct on anti-sense strand at nucleotide position 586 in codon 196. Primers used for analysis on Sense strand were given prefix (S) and for Anti-sense strand (A) will differ at 3’end, which is indicated in red colour. Conserved primers for sense strand were prefix (S) and for anti-sense strand (A).

In the Chapter 4 Prefixes will be not used in the explanation of ARMS-PCR assay and conserved primer sequences were given just for information purpose. These will not be mentioned in the chapter 4.

The site-specific primer sequences are given in Tables 2.10 and Table 2.11. The site-specific primer pairs used for each PCR reaction are given in Tables 2.12 and 2.13.

The PCR conditions designed for this assay are given in Table 2.14. The PCR conditions used vary depending on the primers used. The PCR reaction format for each plasmid is given in Tables 2.15 and 2.16. These PCR reactions were carried out on MJ research, DNA engine, Programmable Thermal Cycler 225 (PTC-225) PCR machine. Dr. Phil Burns, Dept of Pathology, University of Leeds, Leeds, UK designed all the primers sequences and PCR conditions.
Primer pairs used for mutation assays with control plasmid and genetically engineered plasmids, pLS76-2 and pLS76-4 (\(O^6\)-alkylguanine adducts present on sense strand)

<table>
<thead>
<tr>
<th>Type of Plasmid</th>
<th>Anti-sense Strand</th>
<th>Sense Strand</th>
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<tbody>
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<td>SC &amp; A2</td>
</tr>
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<td>AC &amp; S1</td>
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<td></td>
<td>AA &amp; S2</td>
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<tr>
<td></td>
<td>AG &amp; S1</td>
<td>SG &amp; A2</td>
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<tr>
<td>Plasmid 2</td>
<td>AT &amp; S2</td>
<td>SC &amp; A2</td>
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<td>AC &amp; S1</td>
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<td></td>
<td>AG &amp; S1</td>
<td>SG &amp; A2</td>
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<td>Plasmid 4</td>
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<td></td>
<td>AG &amp; S1</td>
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</table>

Table 2.12: Sequence specific primers used for base pairing analysis on plasmid, pLS76-2 containing methylated adduct and plasmid, pLS76-4 containing carboxymethylated adduct on sense strand at nucleotide position 587 in codon 196.

Primer pairs used for mutation assays with control plasmid and genetically engineered plasmids, pLS76-3 and pLS75-5 (\(O^6\)-alkylguanine adducts present on anti-sense strand)

<table>
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<tr>
<th>Type of plasmid</th>
<th>Anti-sense Strand</th>
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<tbody>
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<td>Control</td>
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<td></td>
<td>AG &amp; S1</td>
<td>SG &amp; A1</td>
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<td>Plasmid 5</td>
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<td>SC &amp; A3</td>
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<tr>
<td></td>
<td>AG &amp; S1</td>
<td>SG &amp; A1</td>
</tr>
</tbody>
</table>

Table 2.13: Sequence specific primers used for base pairing analysis on plasmid, pLS76-3 containing methylated adduct and plasmid, pLS76-5 containing carboxymethylated adduct on anti-sense strand at nucleotide position 586 in codon 196.
Optimal PCR reaction conditions designed for mutation assays with different pairs of primers

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</table>

Table 2.14: Optimal PCR conditions designed for ARMS-PCR assays for characterisation of mutations induced by O\textsuperscript{6}-alkylguanine adducts (O\textsuperscript{6}-MedG and O\textsuperscript{6}-CMdG).

PCR Reaction format used for AMS-PCR assays for control and adducted plasmids, pLS76-2 and pLS76-4

<table>
<thead>
<tr>
<th>Type of Plasmid</th>
<th>Anti-sense strand</th>
<th>Sense strand</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Plasmid 2</td>
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<td>2</td>
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<tr>
<td>Control</td>
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<td>2</td>
</tr>
<tr>
<td>Plasmid 4</td>
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</tbody>
</table>

Table 2.15: PCR conditions used for ARMS-PCR assay for determining base pairing at adduct site i.e., nucleotide position 587 on sense strand - Control plasmid, O\textsuperscript{6}-MedG adduct containing plasmid, pLS76 - 2 and O\textsuperscript{6}-CMdG adduct containing plasmid, pLS76 - 4.
**PCR Reaction format used for AMS-PCR assays for control and adducted plasmids, PLS76-3 and pLS76-5**

<table>
<thead>
<tr>
<th>Type of Plasmid</th>
<th>Anti-sense strand</th>
<th>Sense strand</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>2</td>
</tr>
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<td>Plasmid 3</td>
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<td>Control</td>
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<tr>
<td>Plasmid 5</td>
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</table>

Table 2.16: PCR conditions used for ARMS-PCR assay for determining base pairing at adduct site i.e., nucleotide position 586 on anti-sense strand - Control plasmid, O6-MedG adduct containing plasmid, pLS76 - 3 and O6-CMdG adduct containing plasmid, pLS76 - 5.
2.4 Methods used in the determination of Mutation Spectrum of KDA using Human p53 Knock-in (Hupki) Mouse Primary Embryonic Fibroblast Cell lines

2.4.1 Synthesis of Potassium Diazoacetate

Ethyl diazoacetate (1.14g, 1.06ml) was stirred in dark with a solution of potassium hydroxide (1.14g, 2 mol equivalents) in water (11.4ml) until the mixture becomes homogenous (ca. 4h). The resulting solution of KDA (0.9M) was stored at -80°C until required when it is thawed and diluted in water to a suitable concentration and used without further purification. Attempts to purify the material were known to result in extensive decomposition and polymerisation (Kreevoy and Konasewich, 1970). Potassium diazoacetate was characterised by research technician Mr Gerhard Erben at core facilities, German Cancer Research Centre, Heidelberg, Germany using both positive and negative ion mode on ESI Triple Quadrupole; Thermoquest TSQ 7000 Mass spectrometer.

**Reaction:**

\[
\text{(Ethyl diazoacetate)} \quad \overset{\text{+}}{\text{N} \equiv \text{N} = \text{C} = \text{O} - \text{Et}} + \text{KOH} \quad \rightarrow \quad \text{(Potassium diazoacetate)} \quad \overset{\text{+}}{\text{N} \equiv \text{N} = \text{C} = \text{O} - \text{K}^+}
\]

**LC-MS data of Potassium diazoacetate:**

MS (m/z): Positive ion Mode - (M+H\(^+\)) found at 123.96, (M+K\(^+\)) found at 162.8, (2M+K\(^+\)) found at 286.7 and (3M+K\(^+\)) found at 410.8. Negative ion Mode - (M-H\(^-\)) found at 84.9, (2M-2H\(^+\)+K\(^+\))\(^-\) found at 208.8, (3M-3H\(^+\)+2K\(^+\))\(^-\) found at 332.7 and (4M-4H\(^+\)+3K\(^+\))\(^-\) found at 456.7.
2.4.2 Cytotoxicity Tests of KDA on Hupki Mouse Primary Embryonic Fibroblast Cell Lines

Two independent cytotoxicity tests were carried out to determine and confirm the optimal mutagenic dose of potassium diazoacetate. Primary embryonic fibroblasts from Hupki embryos (Trp53<sup>tm1/Holl</sup>, homozygous for the humanised knock-in p53 allele harbouring) were distributed at 2×10<sup>5</sup> cells/per well into six-well plates and grown in DMEM medium supplemented with 10% FCS. Cytotoxicity test 1 consists of one control six-well plate and three individual six–well plates treated with KDA concentrations of 0.1μM, 1μM and 10μM. Whereas, cytotoxicity test 2 comprised one control six-well plate and four individual six-well plates treated with KDA concentrations of 1μmol, 5μmol, 10μmol and 20μmol. Embryonic fibroblast cells were plated and allowed to attach overnight. After 24hrs, cells were washed with PBS and replaced with DMEM medium containing KDA for 1 hour. Treatment was terminated by washing with DMEM medium and replacing with fresh medium. Response to KDA was observed by monitoring cell growth. The sensitivity and percentage viability of cells was monitored at regular intervals of every 24hrs by cell counting on haemocytometer using trypan blue exclusion method.

2.4.3 Cell culture of KDA treated Primary Embryonic Fibroblast Clones of Hupki Mouse

Primary embryonic fibroblasts from Hupki mouse were distributed at 2×10<sup>5</sup> cells/per well into six-well plates and grown in DMEM medium supplemented with 10% FCS. Two independent experiments were set up, of which one set of experiment consists of 8×6-well plates of totally 48 clones incubated with 10μM KDA and the other set containing 4×6-well plates of 24 clones incubated with 100μM of KDA at passage 2.
as described in section 2.4.2. 6×6 well plates were just seeded with $2 \times 10^5$ untreated embryonic fibroblast cells/per well to be used as positive controls. Cultures were passaged at ratios ranging from 1:2 - 1:4 dilutions, until mutant selection and clonal expansion occurred to form a homogenous mutant clone. The genomic DNA was then extracted from the above homogenous clones as mentioned in section 2.4.5 to analyse p53 gene mutations.

2.4.4 p53 Mutation Analysis

KDA induced DNA binding domain (DBD) sequence changes in the p53 gene of HUPKI mouse embryonic fibroblasts were examined by PCR amplification of p53 exons 4-9 using primers defined by Affymetrix (Santa Clara, CA) and direct fluorescent dideoxynucleotide cycle sequencing (Model 310 Genetic analyser) using standard protocols from Applied Biosciences International (Salt Lake City, UT) (Liu et al, 2004). DNA sequencing experiments were carried out by research assistant Mr. Karl Muelbhar in Dept of Genetic Alterations in Carcinogenesis, German Cancer Research Centre, Heidelberg, Germany.

2.4.5 Genomic DNA Extraction from KDA treated Cells for Adduct analysis

The Hupki mouse primary embryonic fibroblasts were exposed in vitro to 10μM, 100μM, 200μM, 300μM, 400μM and 500μM KDA for 24hrs. DNA was extracted from these cells using the following procedure.

1. Cell pellet in FBS medium was transferred to a 1.5 ml microcentrifuge tube and spinned for 2 minutes. Supernatant was decanted.
2. The pellet was resuspended in 500 µl of TE buffer by repeated pipetting. 30µl of 10% SDS and 3µl of 20mg/ml proteinase K were added to the above solution followed by thorough mixing. The reaction mixture was incubated overnight at 37°C on a heat block.

3. An equal volume of phenol/chloroform was added and mixed well by inverting the tube until the phases were completely mixed, followed by centrifugation for 2 minutes.

**CAUTION: PHENOL CAUSES SEVERE BURNS, WEAR GLOVES, GOGGLES, AND LAB COAT AND KEEP TUBES CAPPED TIGHTLY.**

4. The upper aqueous phase was transferred to a new tube and an equal volume of chloroform reagent was added and centrifuged for 2 minutes. The upper aqueous phase was collected and transferred to a new tube.

5. 1/10th volume of sodium acetate was added to the aqueous phase.

6. 0.6ml of isopropanol was added and mixed gently until the DNA was precipitated. The supernatant was carefully decanted.

7. DNA was washed with 1 ml of 70% ethanol for 30 sec until the DNA was precipitated. The supernatant was carefully decanted. DNA was resuspended in 100-200 µl of TE buffer.

8. RNase mix (100 µg of RNase A, or 10U of RNaseT) was added to the above solution and incubated for 30 min at 37°C.

9. 100 µg of Proteinase K was added to the above solution and incubated for another 30 minutes at 37°C.

10. Steps 5 – 7 were repeated again.

11. The concentration of DNA was calculated from absorbance readings obtained at 260nm by diluting 10 µl of DNA into 1 ml of TE (1:100 dilutions).
3.0 INTRODUCTION

DNA is a long chain polymer made up of three basic units: bases (guanine, adenine, cytosine and thymidine), sugar (2'-deoxyribose) and phosphate. DNA can be damaged via a variety of agents by modifying these three parts. DNA is highly susceptible to the chemicals from internal and external sources due to the presence of multiple functional groups such as amine, ketone, double bond and hetero-rings. Synthetic chemistry of base-modified DNA has provided powerful tool for studying DNA-related subjects like mechanisms of mutations, DNA damage and repair. Base modification has an effect on genetic information stored in bases. If these modified bases are left unrepaired, they can result in mutations in important genes like p53 tumour suppressor gene and may give rise to cancer. The recent advances in synthetic chemistry of base-modified DNA and automated synthesis of DNA has provided a powerful tool for studying DNA related subjects such as mechanisms of mutagenesis, DNA damage and repair (Xu, 2002).

Chemical Synthesis of DNA was started in the mid-1950's primarily from Sir Alexander Todd's lab in Cambridge. In 1960, Khorana and his co-workers developed phosphodiester chemistry [Figure 3.1]. The major drawback of this approach was the difficulty of purifying the desired oligomers after each elongation step. This purification was used to be carried out with anion exchange chromatography. Later phosphotriester chemistry is introduced which made purification less tedious, but still took many days to make a short oligomer [Figure 3.1]. With the rapid development of molecular biology, the synthetic chemistry of DNA had to be further improved. In the mid-1980's Merrifield's solid-phase method was adapted for DNA and this eliminated the need for purification of intermediates.
This solid phase approach is coupled with fast reacting phosphoramidites, which allowed rapid synthesis of DNA [Figure 3.1]. Automated DNA synthesis with phosphoramidite chemistry is the method of choice for routine synthesis of DNA in many labs (Xu, 2002).

![Chemical structures](image)

(a) Phosphodiester, (b) Phosphotriester, (c) Phosphoramidite

Figure 3.1: Different types of phosphorous chemistries used in synthesising DNA monomers:

There are two major approaches to synthesize base modified nucleosides. One approach is to synthesise a modified base, and then to join the base with suitably protected deoxyribose moiety via chemical coupling or enzymatic method to produce the base modified nucleosides. The other approach is direct modification of a naturally occurring nucleoside. Chemical approach provides the possibility to link almost any un-natural base or purpose-designed base (e.g. 7-deazadenine) to a suitable deoxyribose precursor to form modified nucleoside. The enzymatic method involves an enzymatic deoxyribosyl transfer reaction to the modified base. Chemical coupling is always problematic. This is because of the instability of the intermediates to the conditions of synthetic transformations, incorrect stereo forms of the products and multiple protecting groups. Comparatively the enzymatic method provides the required isomer (β form) under mild conditions. However, the availability of required enzymes and poor solubility of modified nucleosides is another problem. The
chemical modification can be carried out directly on the existing base of the nucleoside. To modify the base of a nucleoside, the hydroxyl groups of the sugar moiety must be protected to ensure that the reaction takes place site specifically on the base (e.g. $O^6$-methylguanine and $O^4$-methylthymidine) (Xu, 2002).

The major application of base modified nucleosides is in DNA damage studies. DNA is constantly damaged in the cells and modified bases are formed and repaired. To understand these cellular processes it is vital to have base-modified DNA. A classic example is $O^6$-methylguanine. This adducts are formed when methylating agents react with DNA. It can base pair with both cytosine and thymine, hence causing GC to AT transitions. This adduct was found to be repaired by DNA repair protein called $O^6$-methylguanine-methyltransferase. This repair involves transferring the methyl group from the oxygen at the $O^6$-position of guanine to cysteine on the protein. The base modified DNA containing $O^6$-methylguanine and $O^4$-methylthymidine can be used as substrate for this enzyme to study DNA repair process. They can also be used to crosslink with proteins to trap unstable DNA-protein complexes. They are also used for NMR structure analysis of DNA structure or its interaction with proteins. It is a useful tool in constructing a universal base to pair indiscriminately with all four natural bases for mutagenic and recombinant DNA experiments (Xu, 2002).
Chapter 3: Synthesis and Characterisation of $O^6$-alkylguanine adduct containing Oligonucleotides

3.1 Results

3.1.1 Synthesis of $5'-O-(4,4'$-dimethoxytriphenylmethyl)-$N^2$-phenylacetyl-$O^6$-methoxycarbonylmethyl-2'-deoxyguanosine-3'-$O$-(2-cyanoethyl-$N$,$N$-diisopropylamino)-phosphoramidite (compound 7)

$5'-O-(4,4'$-dimethoxytriphenylmethyl)-$N^2$-phenylacetyl-$O^6$-methoxycarbonylmethyl-2'-deoxyguanosine-3'-$O$-(2-cyanoethyl-$N$,$N$-diisopropylamino)-phosphoramidite [compound 7] was synthesised according to reported synthetic route (Xu, 2000) [Section 2.2]. The starting material, 2'-deoxyguanosine was purchased from Cruachem [Transgenomics, Bioconsumables]. All the chemicals used in this synthesis were obtained from either Acros or Fisher Scientific UK Ltd. The exocyclic amino group is protected using phenyl acetyl group. The hydroxyl groups of the nucleoside were protected with acetyl groups, which is cost cost-efficient. Triethylamine in methanol was used for selective removal of the acetyl groups, but not the phenyl acetyl group. This synthesis involves facile sulfonylation of the 6-oxygen of the guanine. The $O^6$-sulfonated derivative is quiet reactive to be displaced by amine and then alkoxide ions. The intermediates [Figure 3.2 (compounds 1-6)] obtained during the synthesis were characterised using both $^1$H NMR [Appendix 1] and LC-MS [Appendix 2] and the final product (compound 7) was characterised using $^{31}$P NMR.

3.1.2 Synthesis of Oligonucleotides containing Modified bases $O^6$-methyl-2'-deoxyguanosine and $O^6$-carboxymethyl-2'-deoxyguanosine.

Modified oligonucleotide synthesis was carried out on Expedite™ 8909 series instruments DNA synthesizer, later deprotected to remove all protecting groups [Section 2.2.2]. All the oligonucleotides were synthesised at a scale of 1μmole [see
Table 2.1 in chapter 2. The yield of the oligonucleotides was estimated from trityl yield curves and by measuring OD_{260} values. Oligonucleotides were initially purified.

**Structures of Compounds 1-7:**

![Chemical structures of compounds 1-7.](image)

**Figure 3.2:** Structures of compounds 1-7. compound(1): 3',5'-diacetyl-2'-deoxyguanosine, compound(2): 3',5'-diacetyl-N^2-phenylacetyl-2'-deoxyguanosine, compound(3): 3',5'-diacetyl-N^2-phenylacetyl-O^6-mesitylenesulphonyl-2'-deoxyguanosine, compound(4): 3',5'-diacetyl-N^2-phenylacetyl-O^6-methoxycarbonylmethyl-2'-deoxyguanosine, compound(5): N^2-phenylacetyl-O^6-methoxycarbonylmethyl-2'-deoxyguanosine, compound (6): 5'-O-(4,4'-dimethoxytriphenyl)-N2-phenylacetyl-O^6-methoxycarbonylmethyl-2'-deoxyguanosine, compound (7): 5'-O-(4,4'-dimethoxytriphenyl)-N2-phenylacetyl-O^6-methoxycarbonylmethyl-2'-deoxyguanosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino)-phosphoramidite.
on NENSORB™ PREP nucleic acid purification columns [Section 2.2.3] followed by further purification on FPLC using Dionex BIOLC system equipped with a DIONEX variable wavelength detector using Pharmacia MonoQ HR 5/5 column equipped with UV detector [Section 2.2.4]. Finally, oligonucleotides were desalted on Sep-pak® C18 cartridges [Section 2.2.5].

3.1.3 Pentamer Analysis

The charge and size differences between site-specifically modified and normal sequences become insignificant as the size of the oligonucleotide increases. This will pose a problem especially when it is required to characterise a site-specifically base modified sequence present in a mixture of normal sequences of the same size. However, it is relatively easy to characterise a site-specifically modified short sequence such as pentamer from other normal pentamers. The standard pentamer AGGCT and modified pentamers AGXCT (X = O₆-MedG adduct) and AGYCT (Y = O₆-CMdG adduct) were synthesised to demonstrate that O₆-alkylguanine adducts were successfully incorporated site-specifically in to oligonucleotides by nucleoside composition analysis. These results can be applied to all other sequences used in this project. All the pentamers have four standard bases A, G, C and T except with pentamers AGXCT and AGYCT, which have an additional modified base. The presence of this modified base creates a charge and size difference when compared with standard pentamer. This charge difference was exploited to prove that all the three pentamers are different. Anion exchange chromatography is good at separating individual pentamers based on charge difference. This analysis was carried out on Dionex BIOLC system equipped with DIONEX variable wavelength detector wavelength detector using Mono Q HR 5/5 column [Section 2.2.6].
given in Table 2.3. The retention times for all the three pentamers were established and then co-injected to demonstrate that all the three pentamers were different. Retention time of pentamer AGGCT is 19.0 minutes [Figure 3.3(a)], AGYCT is 18.0 minutes [Figure 3.3(b)] and AGXCT is 15 minutes [Figure 3.3(c)]. The separation of O^6-CMdG containing pentamer from the one containing O^6-MedG pentamer could be ascribed to the fact that former has an extra charge from the carboxylate anion [Figure 3.3(d)]. At pH 12, the pentamer containing O^6-CMdG has the same number of charges as its parent oligomer containing guanine. However, the later elution of the parent oligomer may be due to its relatively high hydrophobicity compared to O^6-CMdG containing pentamer [Figure 3.3(d)]. Thus, pentamer analysis results indirectly confirms that the sequences used in this project were site specifically modified with O^6-alkylguanine adducts. These results were further supported by the results obtained from the nucleoside composition analysis [Section 3.1.4].
Pentamer Analysis

Figure 3.3: Pentamer analysis was done on DIONEX BIOLC system using Pharmacia MonoQ HR 5/5 FPLC column. Fig 3.3(a) FPLC trace of standard pentamer AGGCT ($R_t$-19.0 min), Fig 3.3(b) FPLC trace of pentamer AGYCT ($R_t$-18.0 min), Fig 3.3(c) FPLC trace of pentamer AGXCT ($R_t$-15.0 min). Fig 3.3(d) FPLC trace of co-injection of three pentamers.
3.1.4 Nucleoside Composition Analysis

Pentamers (1 OD) AGGCT, AGXCT and AGYCT were digested by incubation with nuclease P1 in a solution of pH 4.5 at 37° C and then with alkaline phosphatase in a solution of pH-8.0 for 2hrs at 37° C [Section 2.2.8]. These reactions were followed on Reverse Phase HPLC using Atlantis (5µm, 4.5×25mm) C18 column on Waters™ 616 pump equipped with Waters™ 996 Photodiode Array detector. 0.01M KH2PO4 buffer (pH-6.52)/acetonitrile was used as a mobile phase, monitoring at 260nm at a flow rate of 1ml/min [Table 2.5]. HPLC analysis of standard pentamer AGGCT on digestion with nuclease P1 and alkaline phosphatase gave four peaks that correspond to standard bases 2'-deoxycytidine (Rt - 9.0 min), 2'-deoxyguanosine (Rt - 13.0 min), 2'-deoxythymidine (Rt -14.0 min) and 2'-deoxyadenine (Rt - 16.0 min) [Figure 3.4(a)]. However, with the modified pentamers AGXCT and AGYCT, an additional peak was observed apart from the peaks that correspond to standard bases. This additional peak, (X) was observed at Rr 18.0 min with pentamer AGXCT [Figure 3.4(b)], where as this extra peak (Y) was observed at Rr- 12.0 min with the pentamer AGYCT [Figure 3.4(c)]. The UV profile of peak X and peak Y were quite similar in shape but, different from normal bases. This kind of UV spectrum is typical for O6- substituted compounds. Both the peaks i.e., X and Y exhibit λmax at 247 and 280nm. The UV data and Rt of the peaks X and Y confirms the presence of modified bases X (O6-methyl-2'-deoxyguanosine) and Y (O6-carboxymethyl-2'-deoxyguanosine) in the pentamers. The fluorescence studies provide an additional piece of evidence for the presence of modified bases [Section 3.1.6].
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Figure 3.4: Nucleoside Composition Analysis:

![Graph showing nucleoside composition analysis for pentamer AGGCT.](image)

Figure 3.4(a): HPLC and UV profiles of nucleoside composition analysis on pentamer AGGCT.

![Graph showing nucleoside composition analysis for pentamer AGXCT (X = O-methyl-2'-deoxyguanosine).](image)

Figure 3.4(b): HPLC and UV profiles of nucleoside composition analysis on pentamer AGXCT (X = $O^6$-methyl-2'-deoxyguanosine).

![Graph showing nucleoside composition analysis for pentamer AGYCT (Y = $O^6$-carboxymethyl-2'-deoxyguanosine).](image)

Figure 3.4(c): HPLC and UV profiles of nucleoside composition analysis on pentamer AGYCT (Y = $O^6$-carboxymethyl-2'-deoxyguanosine).
3.1.5 HPLC analysis of purity of mutation assay sequences numbered 10-15.

The sequences numbered 10-15 [Table 3.1] were the sequences that were used for adduct specific mutation assays to study the types of specific mutations induced by modified bases. These complementary sequences comprise the region of p53 cDNA from codons 190-202 with restriction enzymes *BbvC I* and *ApoI* overhanging ends. The sequences i.e., all 25mers and 26mers have same structure on both 5'-end and 3'-ends of modified base sequences. The important prerequisite for mutation assays is to have pure sequences. Impurities could seriously affect the outcome of the mutation assay results, especially if the impurity was more efficient at inducing mutations. Initially, the premise behind this HPLC analysis is to both characterize the 25 and 26mers to verify the presence of $O^6$-alkylated guanine derivatives just like pentamer analysis and to determine the purity of sequences. However, these sequences are much longer and have similar length, which makes the differences of size and charge between individual oligonucleotides insufficiently different to characterise the individual oligonucleotide by observing difference in retention times on C18 columns. Even though standard 25mer and 26mer show differences in retention times with their corresponding 25 and 26mer containing $O^6$-MedG adduct, the same difference can not be demonstrated on HPLC with the 25 and 26mer containing $O^6$-CMdG adduct as observed with pentamer analysis on FPLC. This is because standard 25mer and 26mer were having same charge as their corresponding 25 and 26mer containing $O^6$-CMdG adduct. Hence, this analysis of longer sequences i.e., 25 and 26mers is just limited to determination of purity of sequences. The HPLC analysis of oligonucleotides was carried out on *X-Terra* R M S C18 column (2.5µm, 4.6×50mm) using 0.1M TEAA buffer (pH-7.0)/acetonitrile [Section 2.2.7 and Table 2.4]. Standard 25 and 26mer have retention times 10 and 11 minutes [Figure 3.5(a) and 3.5(b)]. 25 and 26mer containing
$O^6$-MedG adducts have retention time's 12 and 13 minutes [Figure 3.5(c) and 3.5(d)]. Finally 25 and 26mer containing $O^6$-CMdG adducts have retention times 11 and 12 minutes [Figure 3.5(e) and 3.5(f)]. All the oligonucleotides were found to be pure and devoid of any impurities from the HPLC traces.
**Figure 3.5: HPLC Analysis on Mutation assay sequences (10-15) for Purity:**

(a) AU

(b) AU

(c) AU

(d) AU

(e) AU

(f) AU

**Figure 3.5:** HPLC profiles of mutation assay oligonucleotides sequences numbered 10-15. Sequences were purified preparatively on FPLC using Dionex BIOLC system equipped with DIONEX variable wavelength detector. Sequences were analysed on X-terra® C18 HPLC column with specification 2.5μm 50 x 4.6mm. Traces a, c and e correspond to sequences 10, 12, 14 (25mers with G, O⁶-MedG and O⁶-CMdG). Traces b, d and f correspond to sequences 11, 13, 15 (26mers with G, O⁶-MedG and O⁶-CMdG).
3.1.6 Fluorescence Assays

Modified pentamers are very short and simple sequences in which a single modified base is present, apart from four normal bases. Hence, it is quite easy to detect signal from modified base from enzymatically digested pentamer sequence on HPLC. In the case of modified oligonucleotides, which are quite long (12, 25 and 26mers), it is comparatively difficult to detect signal from single modified base. This is because the signal from single modified base is overshadowed by signals from normal bases that are present in large amounts. The oligonucleotides synthesised in this work contains $O^6$-substituted alkylguanines. It was reported previously that $O^6$-alkylguanines are somewhat more fluorescent than the corresponding deoxynucleosides (Gaffney and Jones, 1982). This provides an advantage by allowing the detection of fluorescence signal from single modified base that is present in longer oligonucleotides (12, 25 and 26mers). Melting temperature analysis sequences (sequences numbered from 6-9) and mutation assay sequences (sequences numbered from 12-15) [Table 3.1] were digested with nuclease P1 (pH-4.5) and alkaline phosphatase (pH-8.0) [Section 2.2.9]. The digested samples were analysed on Reverse Phase HPLC using Syngery 4μ, Hydro-RP 80A (150 × 2.00mm) column on Waters™ 2690 Separations Module equipped with Waters™ 996 Photodiode Array detector and Waters™ 474 scanning florescence detector. 0.01M KH$_2$PO$_4$ buffer (pH-6.52)/acetonitrile was used as a mobile phase, monitoring at excitation wavelength 286nm and emission at 378nm at a rate of 0.25ml/min [Table 2.6].

3.1.6.1 Fluorescence Analysis on Sequences numbered 6-9

Sequences numbered from 6-9 were synthesised for melting curve ($T_m$) analysis. Sequences 6 and 7 contain $O^6$-methyl-2'-deoxyguanosine at position X, where as
sequences 7 and 9 contain $O^6$-carboxymethyl-2'-deoxyguanosine at position Y. $O^6$-MedG standard monitored using both fluorescence scanning and photodiode array detector was observed to have $R_t$ -15.0 minutes [Figure 3.6(a) and 3.7(a)]. $O^6$-MedG adduct from the enzymatically digested samples of sequences 6 and 7 were observed to have retention times of 14.932 min [Figure 3.6(b)] and 14.850 min [Figure 3.7(b)] when monitored using fluorescence detector. The UV data was observed to be similar with standard $O^6$-MedG with $\lambda_{\max}$ at 247 and 280. This data confirms the presence of $O^6$-MedG in sequences 6 and 7. An additional piece of evidence, which confirms the presence of $O^6$-MedG, comes from co-injection of standard $O^6$-MedG with digested samples. Both the enzymatically digested sequences 6 and 7 on co-injection with standard $O^6$-MedG adduct gave single peak which were observed to have retention times at 14.938 min and 14.932 min [Figure 3.6(c) and Figure 3.7(c)]. $O^6$-CMdG standard monitored using both fluorescence and photodiode array detector was observed to have $R_t$ - 2.692 minutes [Figure 3.8(a) and Figure 3.9(a)]. $O^6$-CMdG adducts from the enzymatically digested samples of sequences 8 and 9 were observed to have retention times of 3.161 min [Figure 3.8(b)] and 3.194 min [Figure 3.9(b)] when monitored using fluorescence detector. The UV data was observed to be similar with standard $O^6$-CMdG with $\lambda_{\max}$ at 247 and 280. This data confirms the presence of $O^6$-CMdG in sequences 8 and 9. An additional piece of evidence, which confirms the presence of $O^6$-CMdG comes from co-injection of standard $O^6$-CMdG adduct with enzymatically digested samples. Both the sequences 8 and 9 on co-injection with standard $O^6$-CMdG adduct gave single peak which were observed to have retention times at 3.023 mins [Figure 3.8(c)] and 3.194 min [Figure 3.9(c)].
Fluorescence Assay on melting curve ($t_m$) sequence 6 for detection of $O^6$-MedG adduct

**Figure 3.6(a)**

**Figure 3.6(b)**

**Figure 3.6(c)**

**Figure 3.6:** Fluorescence assay on self-complementary melting curve sequence ($t_m$) no-6 containing $O^6$-MedG adduct. Fig 3.6(a) Fluorescent absorbance profile of standard $O^6$-MedG adduct showing $\lambda_{max}$ at 247 and 280 nm. Fig 3.6(b) Fluorescent absorbance profile of nuclease P1 digested, melting curve sequence ($t_m$) no-6 containing $O^6$-MedG adduct. Fig 3.6(c) Fluorescent absorbance profile of co-injected standard $O^6$-MedG adduct and nuclease P1 digested, melting curve sequence ($t_m$) no-6 containing $O^6$-MedG adduct.
Fluorescence Assay on melting curve (t_m) sequence no 7 for detection of O^6-MedG adduct

Figure 3.7(a)

Figure 3.7(b)

Figure 3.7(c)

Figure 3.7: Fluorescence assay on self-complementary melting curve sequence (t_m) no-7 containing O^6-MedG adduct. Fig 3.7(a) Fluorescent absorbance profile of standard O^6-MedG adduct showing λ_max at 247 and 280nm. Fig 3.7(b) Fluorescent absorbance profile of nuclease P1 digested, melting curve sequence (t_m) no-7 containing O^6-MedG adduct. Fig 3.7(c) Fluorescent absorbance profile of co-injected standard O^6-MedG adduct and nuclease P1 digested, melting curve sequence (t_m) no-7 containing O^6-MedG adduct.
Fluorescence Assay on melting curve (t\textsubscript{m}) sequence no 8 for detection of O\textsuperscript{6}-CMdG adduct

Figure 3.8: Fluorescence assay on self-complementary melting curve sequence (t\textsubscript{m}) no-8 containing O\textsuperscript{6}-CMdG adduct. Fig 3.8(a) Fluorescent absorbance profile of standard O\textsuperscript{6}-CMdG adduct showing \(\lambda_{\text{max}}\) at 247 and 280nm. Fig 3.8(b) Fluorescent absorbance profile of nuclease P1 digested, melting curve sequence (t\textsubscript{m}) no-8 containing O\textsuperscript{6}-CMdG adduct. Fig 3.8(c) Fluorescent absorbance profile of co-injected standard O\textsuperscript{6}-CMdG adduct and nuclease P1 digested, melting curve sequence (t\textsubscript{m}) no-8 containing O\textsuperscript{6}-CMdG adduct.
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Fluorescence Assay on melting curve ($t_m$) sequence no 9 for detection of $O^\eta$-CMdG adduct

**Figure 3.9(a)**

**Figure 3.9(b)**

**Figure 3.9(c)**

**Figure 3.9:** Fluorescence assay on self-complementary melting curve sequence ($t_m$) no-9 containing $O^\eta$-CMdG adduct. Fig 3.9(a) Fluorescent absorbance profile of standard $O^\eta$-CMdG adduct showing $\lambda_{max}$ at 247 and 280nm. Fig 3.9(b) Fluorescent absorbance profile of nuclease P1 digested, melting curve sequence ($t_m$) no-9 containing $O^\eta$-CMdG adduct. Fig 3.9(c) Fluorescent absorbance profile of co-injected standard $O^\eta$-CMdG adduct and nuclease P1 digested melting curve sequence ($t_m$) no-9 containing $O^\eta$-CMdG adduct.
3.1.6.2 Fluorescence Analysis on Mutation assay Sequences numbered 12-15

Sequences numbered from 12-15 are synthesised for mutation assays. Sequences 12 and 13 contain $O^6$-methyl-2'-deoxyguanosine at position X, whereas sequences 14 and 15 contain $O^6$-carboxymethyl-2'-deoxyguanosine at position Y. $O^6$-MedG standard monitored using both fluorescence scanning and photodiode array detector was observed to have $R_t$ = 19.569 minutes [Figure 3.10(a) and Figure 3.11(a)]. $O^6$-MedG adduct from the digested samples of sequences 12 and 13 were observed to have retention times of 19.155 min [Figure 3.10(b)] and 19.082 min [Figure 3.11(b)] when monitored using fluorescence detector. The UV data was observed to be similar with standard $O^6$-MedG with $\lambda_{\text{max}}$ at 247 and 280. This data confirms the presence of $O^6$-MedG in sequences 12 and 13. An additional piece of evidence, which confirms the presence of $O^6$-MedG, comes from co-injection of standard $O^6$-MedG with digested samples. Both the sequences 12 and 13 on co-injection with standard $O^6$-MedG adduct gave single peak which were observed to have retention times at 19.913 min and 19.100 min [Figure 3.10(c) and Figure 3.11(c)]. $O^6$-CMdG standard monitored using both fluorescence and photodiode array detector was observed to have $R_t$ = 2.692 minutes [Figure 3.12(a) and Figure 3.13(a)]. $O^6$-CMdG adducts from the digested samples of sequences 14 and 15 were observed to have retention times of 3.161 min [Figure 3.12(b)] and 3.194 min [Figure 3.13(b)] when monitored using fluorescence detector. The UV data was observed to be similar with standard $O^6$-CMdG with $\lambda_{\text{max}}$ at 247 and 280. This data confirms the presence of $O^6$-CMdG in sequences 14 and 15. An additional piece of evidence, which confirms the presence of $O^6$-CMdG comes from co-injection of standard $O^6$-CMdG with digested samples. Both the sequences 14 and 15 on co-injection with standard $O^6$-CMdG gave single
peak which were observed to have retention times at 3.023 min [Figure 3.12(c) and 3.194 min [Figure 3.13(c)].
Fluorescence Assay on mutation assay sequence no 12 for detection of Ω6-MedG adduct

Figure 3.10: Fluorescence assay on Mutation assay sequence no-12 containing Ω6-MedG adduct. Fig 3.10(a) Fluorescent absorbance profile of standard Ω6-MedG adduct showing λmax at 247 and 280nm. Fig 3.10(b) Fluorescent absorbance profile of nuclease P1 digested, mutation assay sequence no-12 containing Ω6-MedG adduct. Fig 3.10(c) Fluorescent absorbance profile of co-injected standard Ω6-MedG adduct and nuclease P1 digested, mutation assay sequence no-12 containing Ω6-MedG adduct.
Fluorescence Assay on mutation assay sequence no 13 for detection of $O^6$-MedG adduct

**Figure 3.11(a)** Fluorescent absorbance profile of standard $O^6$-MedG adduct showing $\lambda_{\text{max}}$ at 247 and 280nm.

**Figure 3.11(b)** Fluorescent absorbance profile of nuclease P1 digested, mutation assay sequence no-13 containing $O^6$-MedG adduct.

**Figure 3.11(c)** Fluorescent absorbance profile of co-injected standard $O^6$-MedG adduct and nuclease P1 digested, mutation assay sequence no-13 containing $O^6$-MedG adduct.

**Figure 3.11:** Fluorescence assay on Mutation assay sequence no-13 containing $O^6$-MedG adduct. Fig 3.11(a) Fluorescent absorbance profile of standard $O^6$-MedG adduct showing $\lambda_{\text{max}}$ at 247 and 280nm. Fig 3.11(b) Fluorescent absorbance profile of nuclease P1 digested, mutation assay sequence no-13 containing $O^6$-MedG adduct. Fig 3.11(c) Fluorescent absorbance profile of co-injected standard $O^6$-MedG adduct and nuclease P1 digested, mutation assay sequence no-13 containing $O^6$-MedG adduct.
Fluorescence Assay on mutation assay sequence no 14 for detection of $O^6$-CMdG adduct

**Figure 3.12(a)** Fluorescent absorbance profile of standard $O^6$-CMdG adduct showing $\lambda_{max}$ at 247 and 280nm.

**Figure 3.12(b)** Fluorescent absorbance profile of nuclease P1 digested, mutation assay sequence no-14 containing $O^6$-CMdG adduct.

**Figure 3.12(c)** Fluorescent absorbance profile of co-injected standard $O^6$-CMdG adduct and nuclease P1 digested, mutation assay sequence no-14 containing $O^6$-CMdG adduct.

**Figure 3.12:** Fluorescence assay on Mutation assay sequence no-14 containing $O^6$-CMdG adduct. Fig 3.12(a) Fluorescent absorbance profile of standard $O^6$-CMdG adduct showing $\lambda_{max}$ at 247 and 280nm. Fig 3.12(b) Fluorescent absorbance profile of nuclease P1 digested, mutation assay sequence no-14 containing $O^6$-CMdG adduct. Fig 3.12(c) Fluorescent absorbance profile of co-injected standard $O^6$-CMdG adduct and nuclease P1 digested, mutation assay sequence no-14 containing $O^6$-CMdG adduct.
Fluorescence Assay on mutation assay sequence no 15 for detection of $O^6$-CMdG adduct

*Figure 3.13(a)* Fluorescent absorbance profile of standard $O^6$-CMdG adduct showing $\lambda_{max}$ at 247 and 280nm.

*Figure 3.13(b)* Fluorescent absorbance profile of nuclease P1 digested, mutation assay sequence no-15 containing $O^6$-CMdG adduct.

*Figure 3.13(c)* Fluorescent absorbance profile of co-injected standard $O^6$-CMdG adduct and nuclease P1 digested, mutation assay sequence no-15 containing $O^6$-CMdG adduct.

*Figure 3.13: Fluorescence assay on Mutation assay sequence no-15 containing $O^6$-CMdG adduct.*

3.13(a) Fluorescent absorbance profile of standard $O^6$-CMdG adduct showing $\lambda_{max}$ at 247 and 280nm.

3.13(b) Fluorescent absorbance profile of nuclease P1 digested, mutation assay sequence no-15 containing $O^6$-CMdG adduct. 3.13(c) Fluorescent absorbance profile of co-injected standard $O^6$-CMdG adduct and nuclease P1 digested, mutation assay sequence no-15 containing $O^6$-CMdG adduct.
3.1.7 Melting curve measurements of modified DNA sequences numbered 4-9

DNA melting curves were obtained by measuring the changes in absorption at 260nm with increasing temperatures. All the melting curves were measured in a solution of 0.1M Hepes, 0.01M NaCl and 0.02M MgCl₂ at pH-7 [Section 2.2.10]. The temperature is increased at the rate of 1° C/min. The melting curves obtained were not ideal s-shaped curves. Hence, the exact melting temperatures were not determined. However, UV absorbance changes plotted against different temperature points and first derivative melting curves have given some insights of stability of different O⁶-alkylguanine base pairs [see table 3.1]. Derivative curve of self complementary sequence 4, indicated that the denaturation of self complementary sequence 4 containing G:T base pair starts around 25° C and have maximum absorbance around 45° C and remain stable there after over a range of temperatures. The possible explanation for this unusual pattern might be due to the problems occurred during reannealing to form hairpin structure by self-complementary sequence 4 containing mismatch base pair G:T [Figure 3.15(a)]. Sequence 5 containing G:C base pair, starts denaturising around 50° C and have maximum absorbance around 70 ° C. [Figure 3.15(a)]. These temperatures relating to the starting point of denaturation exactly corresponds to the temperatures at which increasing in absorbance (inflection points) were observed. Taken together the data of derivative curves and the melting curve (Tₘ) profiles of self complementary sequences 4 [Figure 3.14(a)] and 5 [Figure 3.14(b)] it is clear that sequence 4 containing mismatch base pair G:T is causing strong destabilising effect on hairpin structure than sequence 5 containing G:C base pair. The results obtained from melting curve measurements of self-complementary sequences 6 and 7 in which O⁶-MedG is incorporated to form O⁶-MedG:T base pair [Figure 3.14(c)] and O⁶-MedG:C base pair [Figure 3.14(d)] have confirmed the
Fig 3.14: Melting temperature (Tm) measurements of modified DNA sequences. Traces (a) and (b) Melting curves of G:T (sequence 4) & G:C (sequence 5). Traces (c) and (d) Melting curves of O⁶-MedG:T (sequence 6) and O⁶-MedG:C (sequence 7). Traces (e) and (f) Melting curves of O⁶-CMdG:T (sequence 8) and O⁶-CMdG:C (sequence 9).
Comparison of derivative Tm curve changes between G:T and G:C base pairs

Figure 13.5(a)

Comparison of derivative Tm changes between O6-MedG:t and O6-MedG:C Base pairs

Figure 13.5(b)

Comparison of derivative Tm changes between O6-CMdG:T and O6-CMdG:C Base pairs

Figure 13.5(c)

Figure 13.5: Derivative curves of melting Tm profiles obtained for O\textsuperscript{6}-alkylguanine adducts. Fig 13.5(a) derivative of melting curves of sequence 4 and 5. Fig 13.5(b) derivative of melting curves of sequence 6 and 7. Fig 13.5(c) derivative of melting curves of sequence 8 and 9.
Table 3.1 Melting (Tm) Temperature data of self-complementary sequences containing $O^6$-alkylguanine adducts:

<table>
<thead>
<tr>
<th>Base Pair</th>
<th>Tm($^\circ$ C) at which denaturisation starts</th>
<th>Tm ($^\circ$ C) (App. Values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G:T</td>
<td>25</td>
<td>45</td>
</tr>
<tr>
<td>G:C</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>$O^6$-MedG:T</td>
<td>50</td>
<td>70 - 80</td>
</tr>
<tr>
<td>$O^6$-MedG:C</td>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>$O^6$-CMdG:T</td>
<td>75</td>
<td>n.d.</td>
</tr>
<tr>
<td>$O^6$-CMdG:C</td>
<td>65</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 3.1: Melting (Tm) temperatures at inflection (starting point of denaturation) points of normal 12mer self complementary sequences and modified self complementary sequences containing $O^6$-methyl-2'-deoxyguanosine and $O^6$-carboxymethyl-2'-deoxyguanosine adducts. n.d. denotes not determined.

results obtained previously by Li (Li and Swann, 1989). The observed increase in absorbance (inflection point) plotted against melting temperature (Tm) has confirmed that $O^6$-MedG:T base pair is much more stable, inducing an increase in melting temperature than $O^6$-MedG:C base pair [Figure 3.15(b)]. The derivative curve obtained for the melting temperature profile of the sequence 6 indicated that denaturation of $O^6$-MedG:T base pair starts around 50° C and have maximum absorbance around 70 - 80° C. The denaturation of $O^6$-MedG:C base pair started around 35° C, which can be observed as increase in the absorbance on derivative curve and have maximum absorbance around 60° C. These results are suggesting that
\(O^6\)-MedG adduct predominantly base pairs with T rather than C. The results with sequences containing \(O^6\)-CMdG are not very conclusive. The melting curves obtained from self-complementary sequence 8 containing \(O^6\)-CMdG:T base pair [Figure 3.14(e)] and sequence 9 containing \(O^6\)-CMdG:C base pair [Figure 3.14(f)] suggested that the sequences containing \(O^6\)-CMdG adduct require temperatures higher than 80°C, for melting. Since the maximum temperature used to monitor melting curve is 80°C, it is difficult to extract the exact melting temperature of the above base pairs. Nevertheless, from the temperatures at which raise in absorption (inflection point) was observed on the melting (Tm) curves, it appears that \(O^6\)-CMdG:T base pair is more stable, than \(O^6\)-CMdG:C base pair. These observations are further supported by data obtained from derivative curve of the melting profiles of \(O^6\)-CMdG:T and \(O^6\)-CMdG:C base pairs [Figure 3.15(c)]. The derivative curve of melting profile obtained for CMdG:T base pair clearly indicates that the sequence containing this base pair starts denaturising after 75°C, whereas sequence containing CMdG:C base pair starts denaturising around 65°C. These results, suggests that \(O^6\)-CMdG might preferentially base pair with T. However, the exact temperatures at which they were having maximum UV absorption is not possible to detect from this derivative curves.
3.2 Discussion

Many studies from the laboratory of Miller showed that carcinogenic activity of most chemicals dependent upon their ability to act as electrophiles (Miller, 1978). It was found that some carcinogens are inherently electrophilic, whereas others spontaneously hydrolyse to active species in solution or are activated by cellular enzymes. The resultant reactive intermediates modify the constituents of DNA, RNA, and protein, forming covalent adducts in which the carcinogen residue is joined to nucleophilic atoms of the constituent nucleotides or amino acids. Although all damaged macromolecules can potentially compromise cellular welfare, adducts within DNA have special significance in view of their potential to force replication or repair errors and thus be the chemical progenitors of heritable genetic alterations. It would be interesting to (1) Assess the impact of adduct on local DNA structure and chemistry. (2) Determine the contribution of adduct to the spectrum of mutations induced by chemical or radiation treatment (3) Assess the ability of DNA adduct to act as a cytotoxic lesion. (4) Ascertain the identities of the repair proteins responsible for protecting cells from specific forms of damage (Basu and Essigmann, 1988). Recent advances in the field of nucleic acid chemistry and molecular biology has made it possible to explore the contributory role of DNA adducts to the biological end-points, especially in the progression of cancer disease. Candidate adduct for the above-mentioned studies are identified by using information from three areas. First is the examination of the structure of adducts formed in vivo treatment with a DNA damaging agent that herald a likely change in the Watson-Crick base-pairing. Secondly, examination of spectrum of mutations within a target gene treated with a DNA damaging agent can implicate the base-pairs and DNA sequence contexts that are more prone to mutations. These data in turn provide clues to the identities of the
Chapter 3: Synthesis and Characterisation of \( O^6 \)-alkylguanine adduct containing Oligonucleotides

pre-mutagenic lesions. Finally, facile methods are available in which adduct inserted DNA and RNA templates are used for primer extension assays to determine the type and amount of base mis-incorporation, or termination of chain elongation, can be assessed (Basu and Essigmann, 1988).

![Diagram](image)

**Figure 3.16: Applications of Site-specifically modified Oligonucleotides (Basu and Essigmann (1988)).**

There is a great and increasing interest in the synthesis of oligonucleotides containing carcinogen-modified bases, in particular in those containing \( O^6 \)-alkylguanine because this base is believed to play the important role in the carcinogenic action of N-nitroso compounds. Some of the applications of these sites-specifically modified oligonucleotides are DNA repair, 2D NMR studies, cross-linking and mutagenesis studies [Figure 3.15]. It was found that simple nitrosated bile acid conjugate N-
nitrosoglycocholic acid reacts with DNA to form $O^6$-CMdG and $O^6$-MedG adducts. The latter has already been established as a toxic, pro-mutagenic lesion and is repaired by DNA repair protein, $O^6$-alkylguanine-DNA-alkyltransferase (ATase). It was also reported that $O^6$-CMdG was not repaired when incubated with the same enzyme. This is further confirmed by observation that concomitant methylation and carboxymethylation is the general property of $N$-nitrosoglycine derivatives such as $N$-($N'$-acetyl-L-prolyl)-$N$-nitrosoglycine (APNG) azaserine and diazoacetic acid derivative, potassium diazoacetate (KDA). Information from random mutagenesis studies, using p53 yeast expression assays on KDA, a carcinogen of our particular interest predominantly formed $O^6$-CMdG adduct, which was found to induce mainly GC – AT transitions and some GC – TA transversion mutations in p53 gene (Gottschalg et al., 2006). The preliminary information obtained from the above biochemical studies has suggested that $O^6$-CMdG is an ideal candidate adduct for further investigation on its mutagenic properties. The first step towards the evaluating the genetic and structural effects of an adduct involves its synthesis as part of an oligonucleotide. Generally, three types of synthetic routes are currently available. The first one is total synthesis. This method involves preparation of a protected monomer of the adduct deoxynucleoside [phosphotriester or phosphoramidite] followed by solution or solid phase synthesis of the oligomer by a DNA synthesis protocol. The second method is modification of a preformed oligonucleotide. This involves treatment of an unmodified oligonucleotide with a chemical carcinogen or its activated derivative or with radiation, followed by purification of the desired adducted oligomer. The third method is enzymatic synthesis. DNA polymerase and bacteriophage T4 RNA ligase have been used to build site-specifically modified oligonucleotides biochemically (Basu and Essigmann, 1987). In our studies, total
synthesis was the method chosen for synthesising oligomer containing O\(^6\)-CMdG adduct. The key advantages of this method were high yields, precise adduct placement in the oligonucleotide chain, and, often, better control over final purity than other methods. Fortunately, methods have already been established for O\(^6\)-CMdG adduct to stand against the harsh conditions of synthesis and deprotection, which is a major disadvantage of total synthesis. Traditionally O\(^6\)-substituted base-modified guanines were synthesised either by reaction with diazoalkanes (Friedman et al., 1965) or via the 6-chloro derivative of 2'-deoxyguanosine (Mehta and Ludlum, 1978). The former reaction is cumbersome in that it generates a mixture of alkylated products and hence rigorous chromatographic purification is necessary to obtain the desired product. Although preferable to the diazoalkane approach, the chlorination of the 2'-deoxyribonucleoside can result in extensive depurination. Later, a more convenient route of synthesis involving the facile sulfonylation of the 6-oxygen of the guanine was designed by Gaffney and Jones (Gaffney and Jones, 1982). The O\(^6\)-sulfonated derivative is quite reactive to be displaced by amine and then alkoxide ions. O\(^6\)-CMdG monomer (phosphoramidite) synthesis is primarily based on the preparation of O\(^6\)-arylsulfonate derivative i.e., O\(^6\)-methoxycarbonylmethyl-2'-deoxyguanosine. The acetyl groups, probably more cost-efficient are used instead of methoxyacetyl groups previously used for the protection of 3'-OH and 5'-OH groups of the nucleosides (Harrison et al., 1997). In the conventional synthesis of DNA, an isobutyryl group is generally used for the protection of exocyclic amino group of guanine. However, it has been documented that acyl groups at N\(^2\)-position of O\(^6\)-substituted guanines are much more resistant to removal by ammonia than those at O\(^6\)-unmodified guanine. Therefore, base-labile group such as phenylacetyl group have been chosen to protect exocyclic N\(^2\)-position of guanine. This will allow selective removal of acetyl groups
at 3' and 5'-OH position with out removing phenyacetyl group at $N^2$-position of guanine. The 3',5'-diacetyl-N2-phenylacetyl-2'-deoxyguanosine was first converted in to 3',5'-diacetyl-N2-phenylacetyl-6-mesitylenesulphonyl-2'-deoxyguanosine. This was converted in to quinuclidinium salt, which in turn was replaced by methylglycolate. The acetyl groups protecting the hydroxyl groups of the sugar can be selectively removed by triethylamine in methanol. This treatment did not remove the phenylacetyl group protecting the N2-amino group of the guanine. This procedure is preferred over the alternative deprotection using aqueous NaOH because it is less specific and there is always a high risk of losing N2-phenylacetyl protecting group. Finally, the 3',5'-diacetyl-N2-phenylacetyl-06'-methoxycarbonylmethyl-2'-deoxyguanosine was converted to a monomer by protecting the 5'-OH group with DMT-Cl and 3'-OH group with 2-cyanoethyl-$N,N$-diisopropylchlorophosphoramidite. The characterisation of intermediates using $^1$H-NMR, LC-MS confirmed the presence of phenylacetyl group at exocyclic amino position of guanine base, methoxycarbonylmethyl modification at $O^6$-position of guanine, DMT modification at 5'-OH position of sugar molecule and the characterisation of final compound, $O^6$-CMdG monomer using $^{31}$P NMR confirmed the presence of 2-cyanoethyl-$N,N$-diisopropylphosphoramidite groups. The NMR and LC-MS data was compared with published data confirmed that product obtained was $O^6$-CMdG monomer. All the oligonucleotides that were used in this study are synthesised on an automated DNA synthesiser, Expedite™ 8908 instruments at 1μm scale. The modified oligonucleotides were synthesised using PUSH and PULL method. This method involves interruption of automated DNA synthesis at adducted site and addition of modified DNA adducts on to support manually. The modified adduct in activator solution was added on to DNA chain by gently pushing and pulling adduct containing
solution in the syringe connected to the DNA synthesising support. The modified oligonucleotides containing $O^6$-MedG adduct were synthesised using commercial $O^6$-MedG phosphoramidite. The next step is the deprotection of oligonucleotides. This involves removal of all protecting groups on the sugar molecule as well as on individual bases to obtain normal DNA sequence. Final deprotection of normal oligonucleotides was done by treating with ammonium hydroxide solution overnight at room temperature followed by incubation at $55^\circ$ C for 2 hrs. A disadvantage of the deprotection of oligonucleotides containing $O^6$-MedG by ammonia is that it stabilises the exocyclic amino protecting $N^2$-isobutyryl group of $O^6$-MedG. Hence, $O^6$-alkyl groups are more prone to displacement by ammonia leading to the production of oligomers containing 2,6-diaminopurine. Modified oligonucleotides containing $O^6$-MedG adducts were deprotected by treating with 10% DBU in methanol solution and left in the dark for 5 days. Finally modified oligonucleotides containing $O^6$-CMdg adduct when deprotected with ammonia produced mainly an oligonucleotide containing amide at $O^6$ position of guanine. Hence, these oligomers were deprotected by treating with 0.5M NaOH solution overnight. This method of deprotection will only just hydrolyse the ester group at $O^6$- position of guanine and will not remove $O^6$-carboxymethyl group. This phenomenon was explained by claiming that alkaline solution initially hydrolyses the ester group and the resultant negatively charged carboxylate anion prevents further nucleophilic attack by hydroxide ion on the $O^6$-position of the guanine. All synthetic oligonucleotides will be subjected to some level of contamination depending upon the method of preparation. If they are to be used in NMR studies to evaluate the effect of adduct on DNA confirmation, the purity requirement is not strict. If the oligonucleotides were to be used for genetic studies, the levels of tolerable impurities are very low. Hence, the oligonucleotides that are
used in our studies are initially purified with Nensorb Prep cartridges to remove all reaction intermediates, salts and incompletely short sequences. Then a second purification step was carried out using fast protein liquid chromatography. This is an anion exchange chromatography used to separate impurities from the desired oligomer based on charge difference. Finally the purity of all the oligonucleotides that were synthesised (sequences numbered 1 – 15) were analysed on HPLC. All the oligonucleotides that are to be used in genetic studies observed to be 100% pure from HPLC analysis. Finally, the amount of oligonucleotide obtained after purification was determined by measuring OD values at 260nm on UV Cary photo spectrometer. The characterization of oligonucleotides is very important to verify the presence of modified adducts. The analysis that was routinely used in this project to characterize the oligonucleotides is nucleoside composition analysis. This is an enzymatic reaction in which the oligomers were hydrolysed to individual bases and peaks formed from individual bases will be monitored on HPLC using UV detector. Pentamers (sequences 1-3) were synthesised for this purpose. Since pentamers are short sequences, it is easy to detect signal from modified base when analysed on HPLC with UV detector. All the pentamers that were used in our studies are initially characterised on FPLC. The results of FPLC analysis on pentamers confirmed that all three pentamers were different from each other. This was done by observing the differences in the retention times of individual pentamers separately and by co-injection of all three pentamers together. All the three pentamers were enzymatically digested to individual nucleosides and were analysed on HPLC. Apart from peaks observed with normal nucleosides in pentamer AGGCT, peaks from modified bases $O^6$-MedG and $O^6$-CMdG adducts were observed with modified pentamers AGXCT and AGYCT. The UV spectrum of these modified adducts with $\lambda_{max}$ at 247 and 280
also buttressed the results of nucleoside composition analysis. However, this analysis is practically difficult on longer oligonucleotides. The peak from single modified adduct is overshadowed by peaks from normal bases. It was previously reported that $O^6$-alkylguanines are slightly more fluorescent than the corresponding deoxynucleosides. It is possible to detect signal from modified adduct present in longer oligonucleotides when the samples are analysed with fluorescence detector. The fluorescence assays on longer oligonucleotides (sequences numbered 4-15) have confirmed the presence of modified adducts $O^6$-MedG and $O^6$-CMdG adducts. The peaks obtained from modified adducts have same retention times and UV spectrum as their corresponding standards. Additional evidence confirming the presence of adducts comes from co-injection of the enzymatic digested samples with their corresponding standard $O^6$-alkylguanine adducts gave single peaks. The results of nucleoside composition analysis and fluorescence assays have confirmed the presence of modified $O^6$-alkylguanine adducts in the oligonucleotides that were used for further adduct specific physical and mutagenesis studies. The melting temperature studies on self-complementary sequences have suggested the possible base-pairing preferences of site-specifically incorporated $O^6$-alkylguanine adducts. $O^6$-CMdG adduct was incorporated in two 12mer self-complementary sequences of the type 5' CGC XAG CTT GCG 3' and 5' CGC XAG CTC GCG 3'. Similar sequences containing $O^6$-MedG adduct were used as positive controls. Incorporation of $O^6$-MedG adduct in to the self complementary sequence 7 to form $O^6$-MedG:C base pair has induced only a marginal decrease in melting temperature with respect to normal sequence 5 containing G:C base pair. However, the complementary sequence 6 containing $O^6$-MedG:T base pair has induced a striking increase in melting temperature with respect to normal sequence 4 containing mismatch base pair G:T. It is clear from the data, that $O^6$-
MedG:T base pair caused little destabilisation of hairpin structure formed by self-complementary sequence than that caused by introducing G:T base pair. The above results also support the fact that $O^6$-MedG predominantly induces GC→AT transition mutations due to preferential base pairing with thymidine. The sequences containing $O^6$-CMdG adducts require more than 80° C to destabilise the hairpin structures. Due to the technical limitation of the UV spectroscopy, the melting experiments were not carried out beyond 80° C. Hence, the melting temperatures of self-complementary sequences 8 and 9 containing $O^6$-CMdG adduct were not exactly determined. When the derivative curves of sequence 8 containing $O^6$-CMdG:T base pair compared with sequence 9 containing $O^6$-CMdG:C base pair, it appears that $O^6$-CMdG:T base pair is comparatively stable than $O^6$-CMdG:C base pair. Both $O^6$-CMdG:T and $O^6$-CMdG:C base pairs induced an increase in melting temperature with respect to normal sequence 4 containing mismatch base pair G:T and sequence 5 containing G:C base pair. $O^6$-CMdG:T base pair caused very little destabilising effect than that could be caused by introducing a mismatch base pair G:T. However, when the temperatures at which denaturisation commences were compared between sequences containing $O^6$-CMdG:T and $O^6$-CMdG:C base pairs, it is clear that the sequence 8 containing $O^6$-CMdG:T base pair is much more stable with denaturisation starting around 75° C. The above results are indicating that $O^6$-CMdG might preferentially base pair with thymine. The $O^6$-alkylguanine derivatives have received much attention from the original proposal of loveless that reaction of guanine at the $O^6$-atom would fix the enol tautomer of the base and facilitate the base-pairing with thymine (Loveless, 1969). $O^6$-MedG predominantly induces GC-AT transition mutations. Two possible explanations are that the DNA polymerase might mistake $O^6$-methylguanine for adenine because of the physical similarity between these bases. $O^6$-methylguanine and
adenine are similarly lipophilic and the X-ray crystallography studies on the nucleosides have shown a close similarity in bond angles and lengths between O6-methylguanine and adenine. The second possible explanation is that the important factor in the miscoding is that the alkylG.T mismatch pairs retain the Watson-Crick alignment with N1 of the purine juxtaposed to N3 of the pyrimidine (Swann, 1990). Support for the role of O6-alkylguanines in carcinogenesis initially comes from experiments of Rajewsky. A strong correlation was observed between the incidence of brain tumours in N-ethyl-N-nitrosourea treated rats and the persistence of O6-EtGua in brain DNA (Goth and Rajewsky, 1974). The support for the role of O6-MedG in the carcinogenesis comes from the experiment in which the methylating agent N-methyl-N-nitrosourea induces mammary carcinomas that suffer ras gene mutations of exactly the type caused by O6-MedG adduct (Zarbl et al., 1985). Many in vitro and in vivo studies have suggested a causative role for the O6-MedG in mutagenesis and carcinogenesis. Hence, O6-MedG was chosen by several laboratories as the prototype for synthesis of site-specifically modified oligonucleotides (Borowy-Borowski and Chambers, 1987; Fowler et al., 1982; Kuzmich et al., 1986) and adduct specific mutagenic studies (Bhanot and Ray, 1986; Chambers et al., 1985; Hill-Perkins et al., 1986; Loechler et al., 1984; Topal et al., 1986). From the research findings of the above classic studies it is clear that O6-alkylguanine adducts plays a pivotal role in the carcinogenesis. The observation that nitrosoglycine derivatives and diazoacetate derivatives react with DNA to form O6-MedG, O6-CMdG adducts concomitantly, and the latter is not prone to repair by DNA repair protein, AGT is interesting. In addition, mutagenesis studies on KDA have already indicated that O6-CMdG adduct can induce GC→AT transitions. Hence, it would be particularly useful to study the O6-CMdG adduct specific effects on double helix structure of DNA, type and amount of base
mis-incorporation by this modified base, adduct specific mutagenesis, mutation frequency and sequence context effects on mutagenesis.
Chapter 4: $\beta$-alkylguanine adduct specific Mutagenesis studies

4.0 Introduction

The structurally diverse group of compounds called alkylating agents cause a wide range of biological effects, including cell death, mutation and cancer. These appear to be mediated predominantly by attack at $\beta$ position of guanine. The biological properties and their mode of action have been extensively explored in cell and animal model systems over several decades (Druckrey et al., 1967). Apart from being carcinogenic, these agents are mutagenic, toxic, clastogenic and teratogenic. The spontaneous or metabolical breakdown of these agents in to alkylating species that interact with cellular macromolecules is well-documented (Margison and O’Connor, 1979). These agents react with DNA base’s producing 12 different nitrogen and oxygen adducts as well as phosphotriesters (Margison and O’Connor, 1990; O’Connor et al., 1979). Alkylating agents, damaged DNA contains widely different amounts of 12 alkylated purines / pyrimidines and two phosphotriester isomers. These adducts are found to be substrates for DNA repair proteins (Singer and Hang, 1997). This allowed making a significant progress in establishing a relationship between specific DNA lesions and the biological effects of the agents responsible. Attention has been focused on $\beta$-alkylguanine and, to a lesser extent, $\beta$- alklythymine and 3- alkyladenine and the vast majority of the work has been undertaken on the methyl version of these lesions. The earliest revelations of the toxic and clastogenic effects of the alkylating agents can be a consequence of the formation of $\beta$-methylguanine in DNA were obtained following the isolation of a prokaryotic gene encoding the repair protein $\beta$-alkylguanine-DNA-alkyltransferase.

The presence of $\beta$-MedG has been linked to carcinogenesis via several mechanisms. Most of the attention has been focused on the point mutational effects. G–A transition mutations arise following two rounds of replication
of DNA containing $O^6$-MedG. Several lines of evidence support this. One is the spectrum of associated mutations found in genes that are crucial for malignant transformation. These include H-ras oncogene, in which transition mutations have been reported in codons 12, 13 and 61 (Barch et al., 1991; Rumsby et al., 1991). The transitions are found in a number of locations in tumour suppressor gene p53 (Ohgaki et al., 1992). Sukumar et al., (1983) established that these mutations are dependent on exposure to alkylating agents. They are also dependent on the absence or lack of AGT activity (Estellar et al., 2001; Mitra et al., 1989). Another line of evidence comes from transgenic murine models. Mice, which are over-expressing Atase are more resistant and Atase null mice are more susceptible to carcinogenesis by methylating agents (Kawate et al., 1998; Sakumi et al., 1997). This potential of inducing G-A transitions is also confirmed using both in vitro experiments (Loechler et al., 1984; Safhill et al., 1985) and in vivo experiments using shuttle vector systems (Altshuler et al., 1996).

The possible contribution of recombination mechanism seems to be largely ignored. The contribution of this pathway has not been extensively investigated. There is also experimental evidence to suggest that the toxic effects of this lesion could play a major role in carcinogenesis. The toxic effects of alkylating agents will result in elimination of cells with, presumably highest levels of DNA damage. However, sub-lethally damaged cells, in spite of the presence of $O^6$-MedG in the DNA undergo restorative hyperplasia will replace the dead cells in the tissue to become the predominant population. This will have a substantial effect on genomic stability.

Kyrtopoulos (1998) and Povey (2000) have detected varying levels of $O^6$-MedG in human DNA in several studies on various populations. This may be a consequence of exposure either to known methylating agents that are
Chapter 4: $O^6$-alkylguanine adduct specific Mutagenesis studies

present in cigarette smoke or the diet or to unknown exogenous agents. However, endogenous DNA processes may also contribute to DNA alkylation (Marnett and Burcham, 1993; Hecht, 1999). These endogenous processes include the aberrant methylation of guanine by S-adenosylmethionine and the endogenous nitrosation of compounds containing primary amino groups and their subsequent breakdown to methylating species (Sedgwick, 1997). Shuker and Margison (1997) have showed that N-nitrosated bile acid conjugate, N-nitrosoglycocholic acid is able to methylate DNA in vitro and in vivo. Similar results have been obtained with other nitrosated glycine derivatives like APNG, Azaserine, mesyloxyacetic acid & potassium diazoacetate. It has also been observed that nitrosated glycine derivatives react with DNA giving rise to $O^6$-CMdG and $O^6$-MedG adducts using combined immunoaffinity/HPLC method (Harrison et al., 1999). $O^6$-MedG is well established as a toxic and premutagenic lesion and is a substrate for DNA repair protein, $O^6$-alkylguanine-DNA-alkyltransferase. In contrast, this protein does not repair $O^6$-CMdG. So it is interesting to characterise the type of mutations specifically induced by $O^6$-CMdG adduct in isolation.

4.0.1 p53 Yeast expression Assays

Richard Iggo originally developed the yeast p53 functional assay. This yeast functional assay can be used to determine highly specific mutational fingerprints of carcinogens and mutagens in the human p53 cDNA sequence. Flaman et al. developed a functional assay for the detection of p53 mutations in which human p53 expressed in the haploid Saccharomyces cerevisiae strain yIG397 activates/controls transcription of the ADE2 gene as illustrated in Figure 4.1 (Flaman, 1995). Yeast cells containing wild-type p53 express ADE2, and form white and normal sized colonies
on plates containing limited adenine. On the contrary, cells containing mutant p53 fail to express the ADE2 gene. Because of adenine being limiting for growth, relatively small, red colonies are formed. The red colouring observed in these colonies originates from a build-up of an intermediate metabolite of adenine metabolism.

Figure 4.1: Yeast-based p53 Functional Assay. The ADE2 gene is not expressed if the p53 transactivation function is inactivated by mutation. Transformants are selected on plates that lack leucine but contain sufficient adenine for adenine auxotrophs to grow and turn red due to build-up of an intermediate metabolite. Mutant clones appear as small red colonies against the background of white adenine prototrophs (picture courtesy of P. Burns)

Ishioka has established germ line mutations in p53 by screening patients heterozygous for p53 mutations using functional analysis of separated alleles in Yeast (FASAY) technique (Inga et al., 1997]). Inga has established characteristic mutagenic and lethal properties of CCNU (N-(2-chloroethyl)-N-cyclohexyl-N-nitrosourea) [Inga et al., 1997). Their results suggested that CCNU has a mutation spectrum, which is unique and is consistent with the hypothesis that $O^6$-alkylguanine is the causative lesion. Frebourg has established germ-line mutations in patients with LFS (Li-fraumeni syndrome) using p53 cDNA obtained from lymphocytes of patients (Frebourg et al., 1992). Murata used yeast p53 functional assay to study induction of mutation by nitric
oxide in the p53 tumour suppressor gene and the effect of cytosine methylation on mutation spectrum (Murata et al., 1997).

Initially it was decided to apply the yeast-based p53 functional assay to study the mutagenicity of 0\textsuperscript{6}-carboxymethyl-2'-deoxyguanosine adduct in isolation. This part of the research project was carried out in the labs of our collaborator Dr Philip A. Burns, School of Medicine, Academic Unit of Pathology, University of Leeds. The yeast p53 functional assay described earlier was routinely applied in their laboratory in Leeds to study the p53 mutation spectrum induced by wide variety of mutagens. During the course of mutagenesis studies, it was found that frozen stocks of yeast cells (Saccharomyces cerevisiae strain, yIG 397) were not showing any signs of viability after plating. This strain is also not commercially available. Therefore, transformations in to NOVABLUE E.coli cells standard kit was tried to fix the mutations from 0\textsuperscript{6}-CMdG. Although the principle of this mutation assay is different from p53 yeast expression assays, the important thing here is to fix the mutations from 0\textsuperscript{6}-CMdG in cellular environment. Novablue competent cells (Merck Biosciences) enable convenient, efficient construction of plasmid recombinants. However, BbvCl restriction enzyme over-hanging ends required to attach duplex DNA in to the plasmid is not sticky enough to form large amounts of ligation mixture to transform these cells with adducted plasmid, pLS76. The practicalities of site-specific incorporation of 0\textsuperscript{6}-alkylguanine containing oligonucleotides limit the number of possible sites of insertion. Despite a large number of characterised KDA derivative specific G-mutation sites between codons 120-260 of the p53 sequence, none of these lay within a short sequence between two unique restriction sites, which lies between codons 190-202. These codons fall between restriction enzyme sites BbvCl and Apol. Hence, the above restriction
enzymes were used to construct a plasmid, pLS76 containing single $O^{6}$-alkylguanine adduct. However, religation inefficiency problem of the enzyme $BbvCl$ was not foreseen at this time of research project.

4.0.2 Amplification Refractory Mutation System-PCR Assays

ARMS-PCR is an assay designed to discriminate between target DNA sequences that differ by a single nucleotide in the region of interest. The primers used for this assay differ at the nucleotide that occurs at the extreme 3'-terminus. In a PCR reaction the DNA synthesis step is crucially dependent on correct base-pairing at 3'-end. This difference will enable us to determine the exact base pairing at adducted site by monitoring for primer extension products, which happens only when primer binds to the region of interest. This is similar in principle to primer extension assays, except that in this assay we used double stranded template to double check the nucleotides present on both strands at adducted site to confirm base-pairing unambiguously and the incoming base is already present on the primer at extreme 3'-end. This method is particularly useful in detecting pathogenic mutations [Figure 4.2].

Ultraviolet radiation is the major carcinogenic component of sunlight. UV generates two kinds of pyrimidine dimers in DNA, a cyclobutane dimer and a pyrimidine-pyrimidone photo product. These dimers lead to mutations, notably C to T and CC to TT. The CC – TT mutation is considered to be UV specific mutation. Although there are many reports of a correlation between qualitative exposure to sunlight and non-melanoma skin cancer (NMSC), the relationship with the quantitative exposure is not clear. This is because, it is difficult to measure objectively and exactly how long a person has been exposed to sunlight in their life.
Kawasaki et al., (2000) has tried to exploit the point that UV specific CC-TT mutations are not reversible to develop a method to measure cumulative UV exposure. Unlike other mutation assays, which rely on phenotypic selection of the mutants, they designed mutation assay that is very sensitive and based on genotypic selection. This assay uses site-specifically designed primers (Allele specific primers) to detect mutations at codons 247/8 of the p53 gene of mitochondrial (Mt) DNA in normal skin. Since, CC-TT mutation is a tandem mutation the allele-
specific primers used were having a two-base mismatch to the wild-type sequence. Even though this assay did not show any quantitative correlation between accumulation of the mutation and the level of the UV exposure, but they managed to show a correlation between the presence of mutation in Mt DNA and UV exposure (Kawasaki et al., 2000).

In our current studies, this assay has been used to characterize the $O^6$-carboxymethylguanine specific mutations. Five different types of plasmids were constructed of which, one of them is a control plasmid with no adduct and another two plasmids were having $O^6$-MedG adduct and the remaining two with $O^6$-CMdG adduct incorporated into the p53 cDNA. The p53 cDNA amplified from these plasmids containing adducts were studied with sequence specific mismatched primers using ARMS-PCR assay to characterize the type of specific mutations induced by $O^6$-CMdG and $O^6$-MedG adducts.
4.1 Results

4.1.1 Extraction of Plasmid, pLS76 DNA using QIAfilter plasmid Midikit

Plasmid, pLS76 is a 9kb p53 expression vector, was first described and constructed by Ishioka (Ishioka et al., 1993) [Figure 4.3]. It expresses full length p53 cDNA from the *ADH1* constitutive promoter [Appendix 3]. The vector also contains *CYC1* (terminator) downstream of the p53 cDNA, yeast origin of replication, *CEN6/ARSH4* and *LEU2* as a selectable marker. pLS76 DNA was extracted for genetic manipulations with restriction enzymes *BbvCl, Apol* and *SacI*, which is necessary for incorporation of modified duplex DNA containing $O^6$-alkylguanine adducts ($O^6$-methyl-2'-deoxyguanosine or $O^6$-carboxymethyl-2'-deoxyguanosine). Plasmid purification procedure is an optimized protocol based on the alkaline lysis method of Birnboim and Doly [Section 2.3.1]. The results of plasmid extraction [Figure 4.4] confirms the presence of plasmid DNA in the fractions of plasmid extractions. The two bands in figure 4.4 are super-coiled (lower band) and open circular (upper band) plasmid DNA.

**Figure 4.3:** pLS76 p53 yeast expression vector. The p53 expression vector was first described and constructed by Ishioka et al. [Ishioka (1993)]. The vector harbours human wild-type p53 cDNA under the control of an *ADH1* promoter and the LEU2 gene as a selectable marker (picture courtesy of P. Burns).
4.1.2 Construction of Genetically Engineered Plasmids Containing $O^6$-alkylguanine Adducts

The mutation assay sequences [Table 2.1] synthesised i.e., 25mer (sense strand) containing either (2'-deoxyguanosine or $O^6$-MedG or $O^6$-CMdG adduct) at nucleotide position 587 in codon 196 and 26mer (anti-sense strand) containing (2'-deoxyguanosine or $O^6$-MedG or $O^6$-CMdG adduct) at nucleotide position 586 in codon 196 were the sequences chosen from p53 CDNA, comprises the region from codons 190-202. This sequence is between the restriction sites $BbvCl$ and $Apol$ in p53 cDNA. Initially it was planned that plasmid, pLS76 DNA is digested with restriction enzymes $BbvCl$ and $Apol$ to obtain double stranded plasmid DNA. The 25 and 26mer, which comprises this region, will be annealed to form a duplex DNA with $BbvCl$ and $Apol$ specific cohesive ends. This DNA duplex was planned to be
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incorporated in to the double stranded plasmid DNA using Quick stick$^{TM}$ Ligase kit. Plasmid, pLS76 DNA was digested separately with both the restriction enzymes $BbvCl$ and $Apol$ to cheque if they are unique restriction sites. Unfortunately, it was found that even though, $Apol$ site is a unique site in p53 cDNA, it is not a unique restriction site in whole plasmid, pLS76 DNA. This posed a problem to construct a vector with single $O^6$- alkylguanine adduct. The p53 sequences were designed to construct a duplex DNA containing single $O^6$-alkylguanine adduct, long before the mutagenesis studies were started. Hence, at that point of research project, these problems were not foreseen. Therefore, preliminary experiments of restriction enzyme mapping of whole plasmid DNA were not executed. However, further restriction enzyme analysis was carried out on plasmid DNA to find another restriction site, which is unique in both plasmid DNA and p53 cDNA. Sacl is another unique restriction site found downstream to $Apol$ site. It was then proposed to double digest the plasmid, pLS76 DNA initially with $BbvCl$ and $SaC1$ to obtain a double stranded plasmid DNA (8.5kb approximately) with $BbvCl$ and $SaC1$ specific cohesive ends. Then another intact plasmid, pLS76 DNA will be digested with $Apol$ and $Sac1$ to obtain the 650bp restriction fragment with $Apol + Sac1$ specific cohesive ends. Finally all the three DNA fragments, which means double stranded plasmid DNA (8.5kb), $Apol + Sac1$ restriction fragment and duplex DNA will be ligated together to form an intact plasmid using Quickstick$^{TM}$ ligase kit [Figure 4.5].
Figure 4.5: Genetic Engineering of plasmid, pLS76 to incorporate O\textsuperscript{6}-alkylguanine Adducts

4.1.2.1 Double digestion of Plasmid, pLS76 DNA with Restriction enzymes \textit{BbvC}I and \textit{Sac}I

Plasmid, pLS76 DNA (30\textmu g) was digested with 10 units of each restriction enzyme \textit{BbvC}I and \textit{Sac}I at 37\textdegree C in a water bath. This reaction was repeated to obtain substantial amounts of this fragment for further genetic manipulations [Subsection
2.3.2.1 in Section 2.3.2]. The lane 2 of the gel in Figure 4.6(a) is showing two clear bands formed from double digestion. The band, which is about 8.5(kb) is the double stranded plasmid DNA with \textit{BbvCl} and \textit{SacI} overhanging ends that is required to incorporate duplex DNA and \textit{Apol} + \textit{SacI} restriction fragment (650bp). This 8.5 kb fragment [Figure 4.6(b)] was purified from agarose gel using QIAGEN’s QIAquick gel extraction kit [Subsection 2.3.2.3 in Section 2.3.2].

\textbf{Figure 4.6(a)}: 1\% Agarose Gel profile showing double digestion of Plasmid, pLS76 DNA with Restriction enzymes \textit{BbvCl} and \textit{SacI}. Lanes 1 and 6 contains High Mass DNA Ladder, Lane 2 contains pLS76 + \textit{BbvCl} + \textit{SacI} double digest, Lane 3 contains pLS76 + \textit{SacI} digest, Lane 4 contains pLS76 + \textit{BbvCl} digest, Lane 5 contains plasmid, pLS76 DNA. \textbf{Figure 4.6(b)}: 1\% Agarose gel profile showing purified 8.5kb Double stranded plasmid DNA. Lanes 1 and 6 contain High Mass DNA ladder. Lanes 2-5 contain purified 8.5kb Double stranded plasmid DNA.
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4.1.2.2 Double digestion of Plasmid, pLS76 DNA with Restriction enzymes $ApoI$ and $SacI$

Another fresh batch of plasmid, pLS76 DNA (30μg) was digested with 10 units of each restriction enzyme $ApoI$ and $SacI$. The optimal temperature required for the enzymatic activity of these two enzymes is different. Hence, the plasmid DNA was digested, one at a time with each enzyme [Subsection 2.3.2.2 in Section 2.3.2]. The
lane 5 [Figure 4.7(a)] of the double digest gel shows a clear band around 650bp, which is the Apol + SacI restriction fragment. This 650bp Apol + SaCl restriction fragment [Figure 4.7(b)] was purified from agarose gel using QIAGEN’s QIAquick gel extraction kit [Subsection 2.3.2.3 in Section 2.3.2].

4.1.2.3 Construction of DNA duplex Containing O\(^6\)-alkylguanine Adducts

The DNA sequences [Table 2.1] were chosen from p53 cDNA encompassing the region from codon 190 to 202 of p53 cDNA. These codons fall between restriction enzyme sites BbvCl and Apol. The sequences 10-11 are standard 25 and 26mer, whereas sequences 12-13 are their corresponding methylated counterparts and sequences 13-14 are carboxymethylated counterparts. These complementary sequences were annealed on a PCR heating block to form a duplex with BbvCl and Apol overhanging ends [Subsection 2.3.2.4 in Section 2.2.2]. The DNA was mixed in such a way to form a duplex structure with adduct present on only one of the strands i.e. either on the sense strand of duplex or anti-sense strand of the duplex [Figure 4.8 and Table 2.7]. The duplex DNA was separated from single stranded DNA using 19:1 non-denaturing polyacrylamide gel electrophoresis [Subsection 2.3.2.5 in Section 2.3.2]. The gels are not shown in this result section. This is because DNA fragments are very small, it is often difficult to stain them with ethidium bromide and also the single stranded DNA are not very well stained with ethidium bromide. Hence, gels did not show very clear and tight bands distinguishing double stranded DNA from single stranded DNA. The gels appear like a smear with a blob separating single stranded fragments from double stranded DNA.
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Figure 4.8: Construction of Duplex DNA with $O^6$-alkylguanine Adduct present on either sense or anti-sense strand

\[
\begin{align*}
5' - & \text{CCT CCT CAG CAT TTT ATC CXA GTG GAA GGA AAT TTG CGT-3'} \\
3' - & \text{GGA GGA GTC GTA GAA TAG XCT CAC CTT CCT TTA AAC GCA-5'}
\end{align*}
\]

\(X\) = indicates sites at which $O^6$-MedG or $O^6$-CmDg are inserted on sense and anti-sense strands

Figure 4.8: Complementary sequences 25 and 26mer chosen from P53 CDNA encompass the region from codon 190-202. These sequences lay between restriction sites BbvCl and Apol. The complementary sequences are annealed to form duplex DNA with adduct present either on sense or anti-sense strand.

The oligomers can be radiolabelled with $^{32}$p to distinguish between single stranded sequences from duplex DNA. However, the lack of necessary facilities, equipment and training personnel at the labs of Department of Chemistry, The Open University means that radiolabelling option cannot be opted. In addition, the time allotted for this part of research project had not allowed further radioactive manipulations on DNA sequences. The gel slice containing duplex DNA was excised and purified by using a combination of techniques called CRUSH and SOAK method (Because the
desired fragment of DNA is extracted from the gel band by crushing and soaking in extraction buffer, therefore the method is called CRUSH and SOAK method) and user developed protocol from QIAGEN.

4.1.2.4 Ligation of Double Stranded Plasmid DNA with Apol + SacI (650bp) Restriction fragment and duplex DNA to Construct an Intact Adducted Plasmid.

All the three purified DNA fragments i.e., double stranded DNA with BbvCl + SacI overhanging ends, Apol + SacI restriction fragment and duplex DNA were mixed in appropriate ratios [Subsection 2.3.2.6 of Section 2.3.2] were ligated using Quick-Stick™ ligase kit. The cohesive ends formed by BbvCl are not very sticky and only ≤ 5% of the ends will re-ligate. Hence, 2, 4 and 8 fold amounts of BbvCl + SacI double stranded DNA were used on trial and error basis to find optimal concentration to form enough ligation mixture [Table 2.8]. This procedure was repeated to obtain substantial amounts of ligation mixture for further experiments. Five different plasmids containing O6-alkylguanine adducts including control plasmid with no adduct were constructed. The information on the type of plasmids constructed is given in Table 2.9. The results of the ligation reaction are shown in Figures 4.9(a) and 4.9(b). The 9kb band in lane 3 and 4 of Figure 4.9(a) indicated the successful incorporation of duplex 1 in to control plasmid, pLS76 – 1. The bands in the lane 3 and 4 of Figure 4.9(b) indicated that duplex 2 and 3 containing O6-MedG adducts were successfully incorporated in to plasmids, pLS76 -2 an pLS76 – 3 with respect to control plasmid, pLS76 - 1. The bands in the lane 4 and 5 of Figure 4.9(b) indicated that duplex 4 and 5 containing O6-CMdG adducts were successfully incorporated in to plasmids, pLS76 -4 an pLS76 – 5 with respect to control plasmid, pLS76 - 1.
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4.1.3 Amplification of p53 cDNA from Genetically Engineered Plasmid Constructs containing Single $O^6$-alkylguanine Adducts

The practicalities of site-specific incorporation of $O^6$-alkylguanine containing oligonucleotides limit the number of possible sites of insertion. Ideally we would have liked to insert $O^6$-CMdG adduct at a guanine that was a known site for mutation.
Figure 4.10: 1% Agarose gel profile of amplified p53 cDNA from both normal and adducted plasmid, pLS76 DNA. Lanes 1 and 9 Contain Hyperladder IV, Lane 2 is negative control (No Plasmid DNA). Lane 3 is positive control (pLS76 DNA), Lane 4 contains p53 cDNA from control plasmid, Lane 5 contains p53 cDNA from plasmid, pLS76 - 2 Lane 6 contains p53 cDNA from plasmid, pLS76 - 3, Lane 7 contains p53 cDNA from plasmid, pLS76 - 4, Lane 8 contains p53 cDNA from plasmid, pLS76 - 5.

However, despite a large number of characterised KDA derivative specific G-mutation sites between codons 120-260 of the p53 sequence, none of these lay within a short sequence between two unique restriction sites, which lies between codons 190-202. Codon 196 is the site chosen for insertion of modified adducts both on sense strand at nucleotide position 587 and on anti-sense strand at nucleotide position 586. 25 and 26mers synthesised were complementary sequences, annealed to produce five different types of DNA duplexes with BbvCI and Apol overhanging ends. Apol site
is unfortunately, a unique restriction site in p53 cDNA but not in whole plasmid DNA. Hence, it took an additional double digestion step with another restriction enzyme \textit{SacI}, which in unique in both whole plasmid DNA and P53 cDNA to construct an intact plasmid with adduct. The restriction ends formed by \textit{BbvCI} are not very sticky. Quality control assay of this enzyme claims after 2-fold over digestion with \textit{BbvCI} < 5% of the DNA fragments can be re-ligated. Hence, failure to ligate sufficient material means transfection of adduct containing plasmid in to bacterial cells, fixing mutations induced by DNA adducts is not possible in cellular environment. PCR is a rapid and versatile method for amplifying target DNA sequences present within a source of DNA. Most importantly, it is a useful method to amplify target DNA sequences from little amounts of source DNA. This technique acts in the same way as most other commonly used mutation systems, except for the fact that it is an \textit{in vitro} system. Nevertheless, the important thing here is to fix mutation from the adducted plasmid.

The Principle of the most p53 mutation assays is to fix mutation in the cellular environment and establish these mutated cells by further sub-culturing techniques. Then, DNA will be extracted from these cells for sequencing to detect the mutations in the p53 cDNA. PCR can fix the mutations from adducted DNA in the same way as other mutations systems. p53 cDNA (800bp) from the genetically engineered plasmids containing adducts were amplified using the p53 specific primers. These primers can only amplify the p53cDNA from the intact plasmid. It is not possible to amplify p53 cDNA, unless the three DNA fragments i.e., double stranded DNA (8.5kb), \textit{ApoI} + \textit{SacI} restriction fragments (650bp) and duplex DNA that were ligated form an intact plasmid. The gel [Figure 4.10] shows the p53 cDNA amplified from all the genetically engineered plasmids. This result confirms that all plasmids...
engineered to incorporate adducted duplexes formed an intact plasmid, which otherwise would not be possible to form 800bp p53 cDNA. The p53 primers BB5 and NBE3 were used to amplify p53 cDNA, which comprises about 800bp [Section 2.3.4]. The gel in figure 4.10 shows the p53 cDNA amplified from both the plasmids containing either normal or adducted duplexes that contain \( O^{6}\)-MedG or \( O^{6}\)-CMdG adducts. Lanes 2 contains negative control (No plasmid DNA) and lane 3 contains positive control (cDNA from normal plasmid, pLS76 DNA). The lanes 4 shows the similar band (800bp) obtained from the control plasmid and lanes 5-6 contain 800bp band from plasmids containing \( O^{6}\)-methyladducts i.e., plasmid, pLS76 - 2 and pLS76 - 3 and lanes 7-8 contain 800bp from plasmids containing \( O^{6}\)-carboxymethyl adducts i.e., plasmid, pLS76 - 4 and pLS76 - 5. This amplification was repeated to obtain substantial amounts for further experimentation.

4.1.4 Characterisation of Mutations Induced by \( O^{6}\)-alkylguanine Adducts using ARMS-PCR Assay

p53 cDNA amplified from both normal plasmid and adducted plasmid DNA were analysed using sequence specific primers with ARMS-PCR assay to characterize the mutations induced by \( O^{6}\)-alkylguanine adducts incorporated either on sense or anti-sense strands in codon 196.

4.1.4.1 Characterization of Mutations Induced by \( O^{6}\)-alkylguanine Adducts in Plasmid pLS76 - 2 and pLS76 - 4, at Nucleotide position 587 on Sense strand of p53 cDNA

P53 cDNA amplified from \( O^{6}\)-methyl adduct containing plasmid, pLS76 - 2 and \( O^{6}\)-carboxymethyl adduct containing plasmid, pLS76 - 4 were analysed using sequence
specific primers [Section 2.3.5]. Two separate sets of primers, which differ at nucleotide that occurs at extreme 3'-end were designed for analysis on both sense and anti-sense strands to double check the base pairing at adducted site [Table 2.10]. The PCR reactions were carried out on PTC-225 PCR machine. These reactions were repeated twice to confirm the base-pairing at adducted site unambiguously. The sequence specific pairs of primers for each PCR reaction, PCR conditions and reaction format to analyse base-pairing at adducted site i.e., at nucleotide position 587 on sense strand were given in Tables 2.12 2.14 and 2.15 for both the methylated plasmid, pLS76 - 2 and carboxymethylated plasmid, pLS76 - 4 in material and methods section, chapter 2. All the PCR reactions except with anti-sense strand specific primers T and A, sense strand specific primers A and G were done using PCR reaction condition 2 for both control plasmid and for the adducted plasmids, pLS76 - 2 and pLS76 - 4. The PCR reaction condition 4 was used for the reaction with anti-sense strand specific primer T with plasmid, pLS76 - 2. Where as, PCR condition 7 with anti-sense strand specific primer A and conditions 3 and 8 were used with sense strand specific primers A and G in all the three cases i.e. control plasmid and plasmids, pLS76 - 2 and pLS76 - 4.

PCR amplifications with both control plasmid and adducted plasmids, pLS76 - 2 and pLS76 - 4 gave primer extension products with anti-sense strand specific primer G and sense strand specific primer C that confirms the presence of normal G:C base pairing at nucleotide position 587 in codon 196 [Figure. 4.11(a) and Figure 4.11(b)]. There were also additional primer extension products observed with plasmid, pLS76 - 2, apart from primer extension products with anti-sense strand specific primer G and sense strand specific primer C. Anti-sense strand specific primer A and sense strand specific primer T related primer extension products were
observed with plasmid, pLS76 - 2 containing methylated adduct on sense strand [Figure. 4.11(a)]. This confirms that some of the plasmid 2 constructs have A:T base pair present in the codon 196 at adducted site as expected with DNA repair free system. This A:T base pair in codon 196 at adducted site i.e., at nucleotide position 587 confirms GC-AT transition mutation inductions by the O6-MedG adduct. The results of the PCR assay with plasmid, pLS76 - 4 [O6-CMdG adduct] were different from plasmid, pLS76 - 2 [Figure 4.11(b)]. Primer extension products with anti-sense strand specific primer G and sense strand specific primer C were observed just as with control plasmid and plasmid, pLS76 - 2. Interestingly, they were also some additional primer extension products observed with plasmid, pLS76 - 4. Primer extension products with anti-sense strand specific primer T and sense strand specific primer A were also observed with plasmid, pLS76 - 4. These results were quiet opposite with the results obtained from plasmid, pLS76 - 2. These products prove that some of the plasmid, pLS76 - 4 constructs has T:A base pair substitution at adducted site. This suggests that a GC-TA transversion mutation was induced by O6-CMdG adduct in codon 196 at the adducted site (nucleotide position 587).
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Figure 4.11(a): Agarose gel showing Primer extension products obtained with methylated plasmid, pLS76 - 2

Figure 4.11(a): 2% Agarose gel profile of ARMS-PCR Assay with control plasmid and methylated plasmid, pLS 76 - 2. Lanes H indicate 100bp ladder. Lanes 1-8 are PCR primer extension reactions with sequence specific primers in the order AT, AC, AA, AG, SC, ST, SA and SG. (Prefix 'A'-anti-sense) (Prefix 'S'-Sense strand).

Figure 4.11(b): Agarose gel showing Primer extension products obtained with carboxymethylated plasmid, pLS76 – 4

Figure 4.11(b): 2% Agarose gel profile of ARMS-PCR Assay with control plasmid and carboxymethylated plasmid, pLS 76 - 4. Lanes H indicate 100bp ladder. Lanes 1-8 are PCR primer extension products with sequence specific primers in the order AT, AC, AA, AG, SC, SA, ST and SG. (Prefix 'A'-anti-sense) (Prefix 'S'-Sense strand).
4.1.4.2 Characterization of Mutations Induced by \( O^6 \)-alkylguanine Adducts in Plasmid pLS76 - 3 and pLS76 - 5, at Nucleotide position 586 on Anti-sense strand of p53 DNA

P53 cDNA amplified from \( O^6 \)-methyl adduct containing plasmid, pLS76 - 3 and \( O^6 \)-carboxymethyl adduct containing plasmid, pLS76 - 5 were analysed using sequence specific primers [Section 2.3.5]. Two separate sets of primers, which differ at nucleotide that occurs at extreme 3'-end were designed for analysis on both sense and anti-sense strand to double check the base pairing at adducted site [Table 2.11]. The PCR reactions were carried out on PTC-225 PCR machine. These reactions were repeated twice to confirm the base-pairing at adducted site unambiguously. The sequence specific pairs of primers for each PCR reaction, PCR conditions and reaction format to analyse base-pairing at adducted site i.e., at nucleotide position 586 on anti-sense strand were given in Tables 2.13, 2.14 and 2.15 for both the \( O^6 \)-methyl adduct containing plasmid, pLS76 - 3 and \( O^6 \)-carboxymethyl adduct containing plasmid, pLS76 - 5 in material and methods section, chapter 2.

Surprisingly, ARMS-PCR assay results with both the plasmid, pLS76 - 3 containing \( O^6 \)-MedG and plasmid, pLS76 - 5 containing \( O^6 \)-CMdG adduct in the anti-sense strand were similar [Figure 4.12(a) and Figure 4.12(b)]. Control plasmids gave primer extended products with anti-sense strand specific primer C and Sense strand specific primer G. These results appear contrasting to the results obtained with control plasmids compared against plasmids containing adducts on sense strands. In fact, they are not different, but similar. The products obtained with control plasmids were in other way because, the adduct site analysed for mutation is on anti-sense strand, but not on sense-strand.
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Figure 4.12(a): Agarose gel showing Primer extended products obtained with methylated plasmid, pLS76 - 3

Figure 4.12(b): Agarose gel showing Primer extension products obtained with carboxymethylated plasmid, pLS76 - 5
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It should always be remembered that primer extension products obtained with the plasmids containing adducts on anti-sense strand will appear opposite way and should not be confused during interpretation of results. The C and G primer extension products obtained with control plasmids confirms the normal G:C base pairing at codon 196 i.e., nucleotide position 586. Extra bands present in the control samples might be unamplified and non-specifically amplified p53cDNA impurities [Figure 4.12(a)]. The primer extension products with anti-sense strand specific primer C and sense strand specific primer G were also observed with plasmid, pLS76 - 3 and plasmid, pLS76 - 5. Additional primer extension products were observed with the adducted plasmids, apart from the normal products C and G. The primer extension products with anti-sense strand specific primer T and sense strand specific primer A were observed with both plasmid, pLS76 - 3 and pLS76 - 5 [Figure 4.12(a) and 4.12(b)]. The presence of these products confirms AT base pair substitution mutation at the adducted site in some of the plasmid, pLS76 - 3 and plasmid, pLS76 - 5 constructs. In the other way it means, a GC-AT transition mutation was induced by both the $\theta^6$-MedG and $\theta^6$-CMdG adducts, when they were present on anti-sense strand.
4.2 Discussion

$O^6$-alkylguanine base is believed to play the important role in the carcinogenic action of N-nitroso compounds. It was previously reported concomitant methylation and carboxymethylation is the general property of N-nitrosoglycine derivatives such as APNG and diazoacetic acid derivatives (KDA and MDA). It was found that simple nitrosated bile acid conjugate N-nitrosoglycocholic acid reacts with DNA to from $O^6$-CMdG and $O^6$-MedG adducts. The latter has already been established as a toxic, pro-mutagenic lesion and is repaired by DNA repair protein, $O^6$-alkylguanine-DNA-alkyltransferase. It was also reported that $O^6$-CMdG was not repaired when incubated with the same repair protein. The question, whether the mutagenecity of N-nitrosocompounds is totally because of $O^6$-MedG adducts or it has to do anything in partly with $O^6$- CMdG adduct has not been explored. Random mutagenesis studies, using p53 yeast expression assays on KDA, a carcinogen of our particular interest predominantly formed $O^6$-CMdG adduct, which was found to induce mainly GC–AT transition and some GC–TA transversion mutations in p53 cDNA (Gottschalg et al., 2006). KDA also reacts with DNA to form $O^6$-MedG adducts. Thus it is practically difficult, if not impossible to determine which adduct causes which mutation and why. The best way to answer this question would be by incorporating single $O^6$- CMdG adduct in to a vector to study site-specific mutagenesis of adduct in question. An adduct site- specific study is most useful when it is compared to a random mutagenesis study with its corresponding mutagen/carcinogen. Mutations induced by a particular carcinogen/mutagen can be influenced by DNA sequence context, mutagen/carcinogen dose, level of SOS induction, cell type and other factors. Adduct site-specific mutagenesis studies involves the synthesis of an oligonucleotide that contains an adduct of known structure at defined position and the use of recombinant
techniques to incorporate this oligonucleotide into an autonomously replicating plasmid or viral based vector. A variety of plasmid and phage-based systems have been used to perform adduct site-specific mutagenesis studies in a double stranded vector. The advantages of studying a lesion in double stranded vector is that the system comes as close as possible to resembling genomic replication. In our studies, plasmid based vector, pLS76, which contains p53 cDNA under p53 regulated promoter was used for adduct site-specific studies. The practicalities of site-specific incorporation of $O^6$-alkylguanine containing oligonucleotides limit the possible sites for insertion. Ideally we would have liked to insert $O^6$-CMdG adduct at a guanine that was a known site for mutation. However, despite a large number of characterised G-mutations specific to potassium diazoacetate, none of these sites between codons 120-260 of the p53 sequence lay within a short sequence between two unique restriction sites, which lies between codons 192-200. Codon 196 is chosen for insertion of modified adducts both on sense strand at nucleotide position 587 and on anti-sense strand at nucleotide position 586. The results section 3.1 of chapter 3 has described the procedures of synthesising adduct containing oligonucleotides. It has also discussed the results confirming the presence of adducts in oligonucleotides from the studies of nucleoside composition analysis, fluorescence analysis and melting curve measurement. 25 and 26mers that were synthesised are complementary sequences falling between $BbvCl$ and $ApoI$ recognition sites. The complementary sequences 25 and 26mers were annealed to form both normal and adducted duplex with $BbvCl$ and $ApoI$ overhanging ends. $ApoI$ site is unfortunately a unique restriction site in p53 cDNA but not in whole plasmid DNA. Hence, it took an additional step of double digestion at another restriction site, which is unique in both whole plasmid DNA and P53 cDNA to construct an intact plasmid containing $O^6$-alkylguanine adduct. $SacI$ is
a unique restriction site, which is located at downstream of Apol site. All the three DNA fragments i.e., $BbvCl + Sacl$ double digest (8.5kb), $Apol + Sacl$ (650kb) restriction fragment and duplex DNA containing adduct were ligated using the ligase enzyme to form an intact plasmid. Initially it was proposed to introduce this intact plasmid DNA containing $O^6$-alkylguanine adduct in to some host cell and fix the mutations induced by $O^6$-alkylguanine adduct with in the host cell. The idea was to use p53 yeast expression system, routinely used in the laboratories of academic unit of pathology, school of medicine, University of Leeds. This is a yeast system, which contains ADE2 gene under the control of p53 regulated promoter. Any mutation in a p53 gene interferes with its transactivation function. This is evident by formation of red colonies instead of normal white colonies. Unfortunately, frozen stocks of yeast cells (Saccharomyces cerevisiae strain, yIG 397) were not showing any signs of viability after plating. This strain is not commercially available. Thus, in vivo studies on mutagenesis of $O^6$-CMdG adduct in yeast cells was given up. Later Novablue competent cells (Merck biosciences), were tried for fixing mutations in cellular environment. They enable convenient, efficient construction of plasmid recombinants [Section 2.3.3]. This system also works based on colour change reaction just as p53 yeast expression system. However, for the purpose of this work, the goal was to fix mutation from adduct in cellular environment. The attempts to fix mutations from adduct in cellular host, hampered by re-ligation inefficiency of the restriction ends formed by $BbvC l$ enzyme. Less than 5% of the ends formed by $BbvC l$ can be re-ligated. Hence, the amount of ligation mixture obtained was not enough to transform the cells with ligation mixture. The transfection of ligation mixture in to Novablue competent cells (Merck biosciences) was not successful. Finally, polymerase chain reaction was used to fix mutations. Even though the idea of fixing the mutations in
cellular environment is faltered, the important thing here is to determine mutagenic specificity and types of mutation induced by $O^6$-alkylguanine adducts. PCR is a very sensitive method and can amplify the desired sequences even from the small amounts of the target sources. p53 cDNA (800bp) from the genetically engineered plasmids containing adducts were amplified using the p53 specific primers. These primers can only amplify the p53 cDNA from the intact plasmid. It is not possible to amplify p53 cDNA, unless the three restriction fragments [double stranded plasmid (8.5kb), Apol + SacI restriction fragment (650bp) and duplex DNA] that were ligated form an intact plasmid. The PCR amplification results confirmed that all plasmids engineered to incorporate duplexes formed an intact plasmid, which otherwise would not be possible to amplify 800bp p53 cDNA, that was observed with positive control. p53 cDNA amplified from both the normal and adducted plasmids were analysed using sequence specific primers with ARMS-PCR assay to determine the mutagenic potential of $O^6$-alkylguanine adducts. ARMS-PCR is an assay designed to discriminate between target DNA sequences that differ by a single nucleotide in the region of interest. The primers are designed to differ at the nucleotide that occurs at the extreme 3' terminus. Since, the DNA synthesis step in a PCR reaction is dependent on correct base pairing at 3'-end, this method is particularly useful in detecting pathogenic mutations. Despite of having several disadvantages such as, inability to reflect the natural replication complexes of the cell systems, sequence context constraints, specificity of detecting and measuring point mutation but not any other genetic endpoints, purity of modified oligonucleotides, the in vitro system provide useful insights in to the type of mutations formed by adducts in question. Two separate sets of primers, which differ at the nucleotide that occurs at the extreme 3'-end were designed for analysis on both sense and anti-sense strand to double check
the base pairing at adducted site. Different PCR conditions were designed to avoid unspecific amplification so that background is reduced which otherwise will make results hard to interpret. The comparison of ARMS-PCR assay results of methylated adduct containing pLS76 - 2 and carboxymethylated adduct containing pLS76 - 4 [both plasmids contain adduct on sense strand at nucleotide position 587- (codon 196)] were quiet contrasting. ARMS-PCR assay results with control plasmid has confirmed normal GC base pairing as expected. Additional primer extension products were observed with plasmids, pLS76 - 2 and pLS76 – 4 apart from anti-sense strand specific primer extension product G and sense strand specific primer extension product C, confirming substitution mutations at adducted site. \(O^6\)-MedG adduct from plasmid, pLS76 - 2, induced GC - AT transition at nucleotide position 587. The results with plasmid containing \(O^6\)-CMdG adduct were quiet opposite. The \(O^6\)-CMdG adduct induced GC - TA transversion mutation at nucleotide position 587. PCR condition 1 is a routine PCR program used in many molecular biology laboratories. This is not a very specific program and when used always ends up with non-specific amplifications making the gel look messy. Hence, more stringent conditions were designed that were specific for different primer pairs used in this assay. PCR conditions 7 and 3 were clearly making a striking difference between the types of mutations induced by \(O^6\)-MedG and \(O^6\)-CMdG adducts. At PCR condition 7, plasmid, pLS76 - 2 (\(O^6\)-MedG adduct) was giving the primer extension product with anti-sense strand specific primer A, where as plasmid, pLS76 - 4 (\(O^6\)-CMdG adduct) was not amplifiable. The other PCR conditions 2,3,4,5 and 6 were not able to demonstrate that difference. The similar result was observed with PCR condition 3. At PCR condition 3, \(O^6\)-methyl adduct containing plasmid, pLS76 - 2 was not amplifiable with sense strand specific primer A, where as \(O^6\)-carboxymethyl adduct containing plasmid,
pLS76 - 4 was amplifiable. At PCR conditions 2 and 3 both the plasmids, pLS76 - 2 and pLS76 - 4 were amplifiable with anti-sense strand specific primer T. It was assumed that may be $O^6$-CMdG and $O^6$-MedG adduct are inducing both transition and transversion mutations. But the $O^6$-methyl adduct containing plasmid, pLS76 - 2 was not amplifiable with sense strand specific program A with any of the PCR programs used, eliminating the scope of expecting a GC – TA transversion mutation by $O^6$-MedG adduct. However, in the assay, program 3 is used for this reaction mainly due to two reasons. First, one was to maintain similar conditions with all plasmids to avoid any scope of ambiguity in the interpretation of the results and secondly $O^6$-carboxymethyl adduct containing plasmid, pLS76 - 4 was showing clear difference with control plasmid at this program. At the same time $O^6$-carboxymethyl adduct containing plasmid, pLS76 - 4 was not amplifiable with sense strand specific primer T that, eliminates any scope of expecting a GC-AT transition mutation. Hence, it was assumed that anti-sense strand primer T specific primer extension product, observed with $O^6$-methyl adducts containing plasmid, pLS76 - 2 might be an artefact. Hence, the PCR condition 4 was used for the amplification at which no primer extension product with anti-sense strand specific primer T was observed with plasmid, pLS76 – 2. PCR condition 8 was used with both the plasmids to reduce unspecific amplification with sense strand specific primer G. The results with plasmids, pLS76 - 3 and pLS76 - 5 containing adducts on anti-sense strand i.e., at nucleotide position 586 in codon 196 were quiet different. Control plasmid on PCR reaction with sequence specific primers gave primer extension products with anti-sense strand specific primer C and sense strand specific primer G, which confirms the presence of normal GC base pair at nucleotide position 586 on anti-sense strand. These results look contrasting to the result obtained with control plasmid, analysed against plasmids containing adducts on
sense strand. In fact, they were not different, but similar. This is because, the adduct site that is being analysed with $\mathcal{O}^6$-methyl adduct containing plasmid, pLS76 - 3 and $\mathcal{O}^6$-carboxymethyl adduct containing plasmid, pLS76 - 5 is present on anti-sense strand. It should always be remembered that primer extended products obtained with plasmids containing adducts on anti-sense strand will appear opposite way and should not be confused during interpretation of results. The results were obtained straight away with PCR condition 2 for plasmids containing adducts on anti-sense strand, except with sense strand specific primer T for which PCR condition 6 is used. Both methylated adduct containing plasmid, pLS76 – 3 and carboxymethylated adduct containing plasmid pLS76 – 5 induced GC-AT transition mutations. Many in vitro (Loechler et al., 1984; Safhill et al., 1985; Singer, 1989) and in vivo studies (Altshuler et al., 1996) have unanimously confirmed $\mathcal{O}^6$-MedG pairs with T and induce GC-AT transition mutation in repair free system. Results from this work are in agreement with literature. Interestingly ARMS-PCR assays results showed that $\mathcal{O}^6$-CMdG adduct can induce both transversion and transition mutations. These results are a bit puzzling. The next questions that immediately that comes in to our mind is how can a single adduct can induce multiple mutations? Usually in case of simple alkylating agents, that each adduct principally induces a single kind of mutation and that different mutations are induced by different adducts. For example, $\mathcal{O}^6$-MedG predominantly induces GC-AT transition, $\mathcal{O}^4$-alkylthymidines mainly induces AT-GC transition and $\mathcal{O}^2$-alkylthymidines induces AT-TA tranversions. Two possible explanations are that the DNA polymerase might mistake $\mathcal{O}^6$-methylguanine for adenine and $\mathcal{O}^4$-alkylthymine for cytosine, because of the physical similarity of these bases. $\mathcal{O}^6$-methylguanine and adenine are similarly lipophilic and the X-ray crystallography studies on the nucleosides have shown a close similarity in bond angles and lengths
between \(\text{O}^6\)-methylguanine and adenine, and between \(\text{O}^6\)-alkylthymine and cytosine.

The second possible explanation is that the important factor in the miscoding is that the alkylG .T and alkyl T .G mismatch pairs retain the Watson-Crick alignment with N1 of the purine juxtaposed to N3 of the pyrimidine (Swann, 1990). However, in the case of \(\text{O}^6\)-CMdG adduct, it seems that it is acting more like a bulky adduct that can induce multiple mutations. Previous KDA, carcinogen specific mutagenesis studies using p53 yeast expression assay demonstrated that majority of the mutations induced by \(\text{O}^6\)-CMdG adduct were GC-AT transitions than any other substitution mutations (Gottschalg et al., 2006). The possible mechanism by which \(\text{O}^6\)-CMdG adduct could induce multiple mutations are discussed below. The different conformations formed by \(\text{O}^6\)-CMdG adduct might induce different mutations. The best evidence comes from the studies on the C8-oxo-Gua adduct, which pairs with C when it is anti-confirmation and with A when it is in syn confirmation (Loechler, 1996). \(\text{O}^6\)-CMdG on sense strand might be in a configuration that can form base pair with A with lowest possible energy. Another possibility is that adenine is preferentially incorporated opposite a range of lesions in certain cases, and this preference has been called the “A-rule”. The A-rule is simply a categorization and not an explanation for mutation pattern. DNA polymerase could not be able to determine the base identity from its structural configuration and should simply have incorporated A opposite \(\text{O}^6\)-CMdG base. In addition, the presence of extra charge on \(\text{O}^6\)-CMdG adduct might affect the base incorporation by DNA polymerase. Not all mutagenic events cannot be interpretable in terms of mis-pairing properties, there is always a lesser or greater degree of ambiguity. It also cannot be ruled out that oligonucleotide containing \(\text{O}^6\)-CMdG adduct might have undergone depurination at some stage. This may have led to the formation of abasic site in plasmid resulting in transversion mutation.
Chapter 4: O\textsuperscript{6}-alkylguanine adduct specific Mutagenesis studies

Additional site-specific conformational studies on O\textsuperscript{6}-CMdG adduct, kinetic studies on base pairing properties and sequence context effects on mutagenesis are to be explored to understand the mechanisms by which O\textsuperscript{6}-CMdG adduct induces multiple mutations.
5.0 INTRODUCTION

The p53 tumour suppressor gene is at the forefront of cancer research because it is the most commonly mutated gene in human cancers. The p53 mutation spectrum provides clues to the etiology and molecular pathogenesis of cancer disease. This protein is clearly a vital component in biochemical pathways leading to carcinogenesis. Both the missense mutations and loss of p53 protein by non-sense or frameshift mutations provide a selective advantage for clonal expansion of preneoplastic and neoplastic cells. One explanation for the commonality of p53 mutations in human cancer is the potential for a missense mutation to cause loss of tumour suppressor function and gain of oncogenic activity, i.e., both endogenous and exogenous carcinogens as well as biological processes are known to cause mutations in the p53 gene. Classes of DNA damage include deletion, substitution, transition (change of a pyrimidine to pyrimidine or a purine to a purine) or transversion (change of a pyrimidine to a purine or vice versa). Chemical carcinogens and their metabolites induce mutations by forming covalent adducts with the nucleotides in DNA. Some pre-mutagenic lesions such as $\text{O}^6$-methylguanine formed due to attack of alkylating agents on DNA, may cause DNA polymerase to misread the base pairing due to the altered hydrogen bonding of a base, which contains additional methyl or ethyl group. A well documented line of evidence established that the most common mutations caused by alkylating agents are GC-AT transitions, consistent with $\text{O}^6$-methylguanine mispairing with thymine. Bulky carcinogen-DNA adducts may render the bases unreadable and stall the replication machinery. DNA polymerases fill these non-instructive sites preferentially with adenine. This leads to GC-TA transversions. Many studies using prokaryotic or eukaryotic cells and site-specific mutagenesis assays have shown that some carcinogenic agents produce fingerprints i.e., specific types and
locations of adducts have been linked with the mutation spectrum of an agent (Greenblatt et al., 1994).

One of the goals of carcinogenesis research is to identify the precise molecular alterations responsible for neoplastic transformation, abnormal differentiation, and growth control. Molecular epidemiology has made an attempt to integrate traditional epidemiological investigation of cancer risk factors with the substantial expansion of knowledge of the molecular mechanisms of the cellular processes. Mutation spectrum analysis is the study of types and locations of DNA alterations. It describes the characteristic patterns of DNA changes caused by endogenous and exogenous mutagens. These alterations represent the interactions of carcinogens with DNA and cellular DNA repair processes. They also reflect the selection of those mutations, which provide premalignant and malignant cells with a growth advantage. Animal models provide opportunities for mutation spectrum studies that cannot be performed with human tumours. The carcinogen-induced mutations in cancer related and house keeping genes can be compared in rodent and human cells [Appendix 4]. This is useful to assess the relevance of these model systems to human carcinogenesis (Greenblatt et al., 1994).

The P53 tumour suppressor gene has very high frequency of point mutations in human tumours [Appendix 5]. The locations of inactivating tumour mutations are numerous and dispersed. In contrast to other tumour suppressor genes, about 87% of the somatic p53 mutations are missense mutations that lead to amino acid substitutions in proteins thus altering protein conformation. Approximately 13% are complex mutations and short insertions or deletions. More than 90% of all mutations identified today have been observed in the DNA binding domain and mainly affect amino acids in highly conserved regions. The residues most commonly
Chapter 5: Studies on KDA Mutation Spectrum

**Codon distribution of p53 Somatic Mutations:**

![Codon distribution of somatic p53 mutations according to version of R5 of the IARC p53 mutation database (Hernandez-Boussard and Hainui, 1998).](image1)

**Figure 5.1:** Codon distribution of somatic p53 mutations according to version of R5 of the IARC p53 mutation database (Hernandez-Boussard and Hainui, 1998).

**Codon distribution of p53 Somatic Mutations:**

![Codon distribution of germ-line p53 mutations according to version of R10 of the IARC p53 mutation database [www-p53.iarc.fr].](image2)

**Figure 5.2:** Codon distribution of germ-line p53 mutations according to version of R10 of the IARC p53 mutation database [www-p53.iarc.fr].
mutated (codons 175, 245, 248, 249, 273 and 282) represent about 30% of all somatic mutations [Figure 5.1] (Hainut and Hollstein, 2000). With the exception of codon 245, five of the six most frequently mutated codons encode arginine residues. These codons play distinct roles in protein-DNA interactions either via directly contacting DNA (residues 248 and 273) or via contributions to the stability of the DNA binding surface (residues 175, 249 and 282) (Cho, 1994). Hotspots at codon 175, 248 and 273 account for approximately 21% of all mutations. This high rate can be explained in part by the presence of a CpG dinucleotide in these residues. CpG dinucleotides are known to be frequently methylated in the vertebrate genome increasing the probability of mutations at such sites. The high frequency of C→T transitions found at CpG dinucleotides in general is believed to result from spontaneous deamination of 5-methylcytosine. Thymidine is formed via deamination of 5-methylcytosine thus resulting in a G:T mismatch, which, if not repaired, creates a C→T transition. (Ehrlich, 1981; Hussain and Harris, 1998). On average these mutations account for approximately 25% of tumour mutations in all human malignancies. The prevalence of GC→AT transitions at CpG sites is higher in internal cancers such as colorectal cancer (~50%), whereas in patients with head and neck cancer, mutations in CpG sites are relatively uncommon (Sidransky and Hollstein, 1996; Moll and Schramm, 1998).

According to the R10, IARC database contains 283 germ-line mutations of which 85% are point mutations and approximately 15% are complex mutations and short insertions or deletions. Residues most commonly mutated are codons 133, 152, 153, 175, 213, 233, 245, 248, 273, 282, 308 and 337 [Figure 5.2].

Many studies, which induced p53 mutations experimentally in mice that are exposed to carcinogens, have not contributed significantly in understanding mutagenic mechanisms and origin of mutations in human cancers. The
experiments with the standard laboratory murine models used to generate the mutation pattern data from the mouse p53 genes. The experiments are clearly heterogeneous. This approach has limitation of species difference in the p53 gene between mouse and human sequences, which restrict interpretation of the distribution of mutations along the gene and their accumulation at preferential locations. This makes the process of inter-study comparisons and conclusions that can be drawn regarding the frequency of p53 mutations complicated. Hence, an experimental model is needed to induce p53 mutations in human p53 gene in vivo (Greenblatt et al., 1994).

**Figure 5.3: The Hupki mouse: a new biomedical research tool (Zelinski et al., 2002).**

Recently, Gene-targeting technology has allowed development of a new mouse model, which explores experimentally the endogenous and environmental factors that may contribute to neoplastic disease in humans. This mouse model is called human p53 knock-in mouse (Hupki) in which the core domain of the endogenous murine p53 allele has been replaced with the homologous, normal human p53 sequence. The exons 4-9 of the murine endogenous p53 gene are replaced with the homologous human sequences. This is a very useful model in comparing the frequency of mouse mutations at specific positions in the p53 gene following the
carcinogen exposure directly with the human data. The mutation pattern in the p53 gene provides clues on the environmental agents or endogenous biological processes that generate these mutations. The other applications of Hupki mouse are that it is also useful in investigating the role of apoptosis in mice and humans as well as in drug screening [Figure 5.3] (Zielinski et al., 2002).

It was observed that when epidermal cells of Hupki mouse irradiated with single acute dose of UVB light, they accumulate UV photoproducts at the same location as p53 gene of human cells. DNA preparations from 2 cm² sections of chronically irradiated Hupki epidermis harbour C to T and CC to TT mutations at two hotspots identified in human cancer, one at codons 278-279 and one at codons 247-248 (Liu et al., 2004). Thus Hupki mouse offers an in vivo approach to such investigations. As we already know that nitrosoglycine derivatives react with DNA to give rise to modified O^6^-alkylguanine adducts. It is also known that this adducts induce GC-AT transition mutations in p53 tumour suppressor gene. Hence, it would be interesting to study the mutation spectrum of nitrosoglycine derivative, potassium diazoacetate (KDA) in p53, tumour suppressor gene using the above model. Potassium diazoacetate is particularly interesting because it is a stable nitrosoglycine derivative. This mouse model was used in this project to study the types and locations of mutations induced by nitrosoglycine derivative, potassium diazoacetate to understand the mechanisms of mutations by which this carcinogen induces mutations. This part of the research project was carried out in the labs of our collaborator Dr Monica Hollstein, Dept of Genetic Alterations in Carcinogenesis, German Cancer Research Centre, Heidelberg, Germany. The above-mentioned murine model studies were routinely carried out in their laboratory to study the p53 mutation spectrum induced by wide variety of mutagens.
5.1 Results

5.1.1 Synthesis and Characterisation of Potassium diazoacetate (KDA)

Potassium diazoacetate was synthesised according to reported synthetic route (Kreevoy and Konsewich, 1970) [Section 2.4.1]. The starting material ethyl diazoacetate (Catalogue No: E-22201) was purchased from Sigma-Aldrich. Ethyl diazoacetate (1.14g, 1.06ml) was stirred in dark with a solution of potassium hydroxide (1.14g, 2mol equivalents) in water (11.4ml) until the mixture becomes homogenous (ca. 4h) [Figure 5.4]. The resulting solution (0.9M) was stored at -80°C until required when it is thawed and diluted in water to a suitable concentration and used without further purification. Attempts to purify the material are known to result in extensive decomposition and polymerisation. The potassium diazoacetate was freeze-dried to obtain a fine yellowish powder and was characterised using LC-MS.

\[
\text{Reaction: } \quad \begin{array}{c}
\tilde{\text{N}}=\tilde{\text{N}}=\text{CH}_-\text{C}_-\text{O}_-\text{Et} + \text{KOH} \\
\text{(Ethyl diazoacetate)} \\
\end{array} \rightarrow \begin{array}{c}
\tilde{\text{N}}=\tilde{\text{N}}=\text{CH}_-\text{C}_-\text{O}_-\text{K}^+ \\
\text{(Potassium diazoacetate)} \\
\end{array}
\]

Figure 5.4: Synthesis of Potassium diazoacetate (Kreevoy, 1970).

5.1.2 Results of Cytotoxicity tests of Potassium diazoacetate

Potassium diazoacetate is particularly interesting because it is a member of family of nitrosated glycine derivatives which decompose or rearrange to give carboxymethylating agents resulting in the formation of $O^6$-carboxymethyl-2'-deoxyguanosine. Two independent cytotoxicity tests were performed to determine and confirm the optimal mutagenic dose of potassium diazoacetate [Section 2.4.2]. Qualitative data on sensitivity and percentage cell survival for controls and the KDA
Toxicity Tests of Potassium diazoacetate (KDA) on *Hupki* fibroblast cells (HUF):

![Graph 1](image1)

**Figure 5.5:** Cytotoxicity Test 1 of potassium diazoacetate (KDA) on Hupki mouse embryonic fibroblast cells. Controls were treated with DMEM medium supplemented with 10% FCS without any KDA. All the cells were exposed to KDA treatment for 24 hrs. Cell viability was monitored for 96 hrs at regular intervals of 24 hrs by cell counting.

![Graph 2](image2)

**Figure 5.6:** Cytotoxicity Test 2 of potassium diazoacetate (KDA) on Hupki mouse embryonic fibroblast cells. Controls were treated with DMEM medium supplemented with 10% FCS without any KDA. All the cells were exposed to KDA treatment for 24 hrs. Cell viability was monitored for 114 hrs at regular intervals of 24 hrs by cell counting.
Percentage Viability of HUF cells treated with KDA

Figure 5.7: Percentage viability of HUF cells calculated from the results of cytotoxicity Test 1 of potassium diazoacetate (KDA) on Hupki mouse embryonic fibroblast cells. Survival percentage represents total number of cells present in the samples treated with KDA divided by total number of cells present in the control samples at corresponding time point.

Figure 5.8: Percentage viability of HUF cells calculated from the results of cytotoxicity Test 2 of potassium diazoacetate (KDA) on Hupki mouse embryonic fibroblast cells. Survival percentage represents total number of cells present in the samples treated with KDA divided by total number of cells present in the control samples at corresponding time point.
Table 5.1: Quantitative data of cytotoxicity test 1 showing sensitivity and percentage survival of HUF cells treated with KDA

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5.1(a)

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5.1(b)

Table 5.1(a): Cell growth data of cytotoxicity test 1. Growth is monitored by cell counting to determine the sensitivity of cells to different concentrations of KDA. Table 5.1(b): Percentage viability data of cytotoxicity test 1. Percentage viability is calculated to determine KDA concentration that reduced the number of cells to 50% of control (IC₅₀).

treated samples of two independent cytotoxicity experiments are displayed in figures 5.5, 5.6, 5.7 and 5.8. Percentage cell survival represents the total number of cells obtained for KDA treated samples divided by number of cells in control plates. Figure 5.5 and Figure 5.6 shows the cell survival as measured by counting the number of cells from samples treated over the dose range of 0 – 10 μm and 0 – 20 μm at regular intervals of 24 hrs. The viability of cells was monitored at regular intervals of every 24 hrs by cell counting for up to 96 hrs in test 1 and 144 hrs in test 2. Figures 5.7 and 5.8 shows percentage cell survival as measured by dividing the total number of cells obtained for KDA treated samples at each time point by total number of cells present in the control samples with respect to that particular time point. A dose dependent
increase in sensitivity and decrease in the percentage of the cell survival was observed from both the experiments.

Table 5.2: Quantitative data of cytotoxicity test 2 showing sensitivity and percentage survival of HUF cells treated with KDA

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5.2(b)

Table 5.2(a): Cell growth data of cytotoxicity test 2. Growth was monitored by cell counting to determine the sensitivity of cells to different concentrations of KDA. Table 5.2(b): Percentage viability data of cytotoxicity test 2. Percentage viability was calculated to determine KDA concentration that reduced the number of cells to 50% of control (IC50).

Ideal toxic dose is the one that reduces the number of viable cells to 50% with respect to control. In cytotoxicity experiment 1, for 10μM KDA treatment a survival rate of 57 and 45% were observed at 48 hrs and 72 hrs, whereas survival rate is almost 100% for 0.1 and 1μM KDA treatments with respect to control [Table 5.1(a) and 5.1(b)]. The above data shows that the sensitivity of HUF cells to KDA was increased 43 and
55-fold following incubation with 10μM KDA by 48 and 72 hrs. HUF cells treated with 0.1μM KDA had not shown any signs of sensitivity, whereas cells treated with 1μM KDA showed 15-fold increase in sensitivity at 48 hrs and relatively no signs of sensitivity at 72hrs. The results from the above data indicate that concentration of 10μM KDA is an ideal IC$_{50}$ concentration to carry our subsequent mutagenesis studies. However, the above data is further analysed by comparing the results from another set of experiments. This set of experiments excluded 0.1μM KDA treatments, but additionally included 5μM and 20 μM KDA treatments to determine exact IC$_{50}$ dose of KDA. The results from second set of experiments are slightly varying from the results obtained from the first set of experiment.

In cytotoxicity experiment 2, for 10μM KDA treatment a survival rate of 91 and 69% compared to 57 and 45% was observed at 48 hrs and 72 hrs. However, the survival rate of HUF cells incubated with 10μM KDA is 54 and 59% at 96 and 144hrs. The survival rate of cells treated with 20μM KDA treatments are 53 and 34% at 48 and 72hrs dropping to 18% by 96 hrs. The survival rates of cells incubated with 1μM and 5μM KDA is almost 100% up to 72hrs there after dropping down to 86 and 78% by 96hrs. In other words the sensitivity of HUF cells was increased by 9, 31 and 46-fold in test 2 compared to 43, 56 and 70- fold in test 1, following incubation with 10μM KDA for 48, 72 and 96 hrs. Whereas, cells treated with 20μM KDA showed 47, 66 and 92-fold increase in sensitivity at 48, 72 and 96hrs. Cells treated with 1 and 5 μM KDA showed 14 and 24-fold sensitivity after 96hrs with respect to control [Table 5.2(a) and 5.2(b)].

Even though toxicity test 2 indicated cells incubated with 20μM KDA showed better toxicity profiles than cells treated with 10μM KDA by 48hrs, the percentage cell survival of cells incubated with 20μM was drastically dropped down
to 34 and 18% compared to 69 and 54% of cells incubated with 10μM KDA by 72 and 96hrs. The toxic effects observed with 10μM KDA concentrations from both sets of experiments are progressive rather than sudden drastic changes observed with cells treated 20μM KDA concentrations. In other words, toxic effects of 20 μM KDA concentration that were monitored by increase in cell sensitivity and decrease in percentage cell survival were severe compared to 10μM KDA concentrations. The cell sensitivity and percentage cell survival of cells incubated with 10μM KDA were moderate enough to obtain mutant clones. Hence, subsequent experiments were carried out with 10μM KDA treatments to obtain mutants for p53 analysis.

5.1.3 Results of p53 Mutation Analysis

Primary embryonic fibroblasts from *Hupki* embryos (Trp53<sup>tm1/Holl</sup>, homozygous for the Knock-in p53 allele harbouring human p53 sequences) were distributed at 2×10<sup>5</sup> cells/per well into 6-well plates and grown in DMEM medium supplemented with 10% FCS. Two independent experiments were carried out of which one experiment consisted of totally 48 clones exposed to 10μM of KDA and the other experiment consisted of 24 clones exposed to 100μM of KDA at described in section 2.4.2. 36 clones of HUF cells that were not subjected to any treatment were used as positive controls. Initially cultures incubated with 10μM KDA did not show enough signs of toxicity. Due to the highly unstable nature of KDA, the toxicity profiles of this compound are varying from experiment to experiment. It is assumed that KDA might be decomposing very fast when mixed with the culture medium. Hence, another experiment was set up with clones treated with KDA concentration increased by 10-fold. This experiment was set up as a backup, just in case, that if any chance to obtain mutant clones from cultures treated with 10μM KDA were hampered. Cultures were
Chapter 5: Studies on KDA Mutation Spectrum

passaged at ratios ranging from 1:2 to 1:4 dilutions until clones survived the stress period to form morphologically homogenous clones. Genomic DNA was then extracted from these morphologically homogenous clones as described in section 2.4.5. DNA binding domain (DBD) sequence of the p53 gene were examined by PCR amplification of p53 exons 4-9 using fluorescent dideoxynucleotide cycle sequencing using agents, primers, standard protocols, and equipment (Model 310 Genetic analyser) from Applied Biosciences International (ABI, Salt Lake City, UT), essentially as described previously [Section 2.4.4]. The genomic DNA of some of the mutant clones that were obtained from cultures incubated with 10μM KDA was sequenced to detect the presence of any functional mutations in the DBD region of p53 gene. The results did not show the presence of any functional mutations in the DNA binding region of p53 gene (exon 4 – 9). It was assumed that KDA treated clones might have incurred spontaneous mutations by secondary mechanism at some other down stream targets of p53 gene, which lead to the formation of homogenous clones that survived the crisis period. Hence, further sequencing of the remaining clones was not carried out.
5.2 Discussion

Several studies have pinpointed the fact that p53 plays a central role in tumorigenesis both in humans and in experimental systems. Since 1989, about 570 different p53 mutations have been identified in more than 8000 human cancers. The prevalence of p53 mutations varies among tumour types ranging from 0 to 60% in major cancers and is over 80% in some histological subtypes [Appendix 6] (Greenblatt et al., 1994). M. Hollstein and C.C.Harris initiated a database of these mutations in 1990. This database originally considered of a list of somatic point mutations in the p53 gene of human tumours and cell lines compiled from published literature and made available in a standard electronic form. The database is maintained at the International Agency for Research on Cancer (IARC) and updated versions are released twice every year (January and July) (Hainut et al., 1998). To date as per R10 released in July 2005 from IARC the database contains 21,587 somatic mutations [appendix 7] reported in 1,876 original publications, 283 germ-line mutations [appendix 8] reported in 129 publications (published between 1989 and December 2004) and functional information on more than 425 mutant proteins. The database is available in different electronic formats at IARC (http://www.iarc.fr/p53/homepage.htm) or from the European Bioinformatics Institute (EBI) server (http://www.ebi.ac.uk) (Hainut et al., 1998). A large and diverse collection of human tumour mutations at a specific locus of p53 provides information of mutation spectra of environmental or endogenous biological factors that elicit genetic damage and development of cancer (Hainut and Hollstein et al., 2000). P53 gene is well suited to mutations spectrum analysis for several reasons, (1) since p53 mutations are common in many human cancers (2) Its modest size (11 exons, 393 amino acids permits study of the entire coding region. (3) Point mutations that alter p53 function are distributed over a large region of the
molecule (Greenblatt et al., 1994). Mutations appear because of endogenous processes or of the action of exogenous, physical or chemical carcinogens. Endogenous processes include methylation and deamination of cytosine at CpG residues, injury by free radicals generated by biological processes and errors in DNA-repair and synthesis. Mutations do not form at an equal rate at all base positions. For example, formation of adducts in p53 DNA by metabolites of benzo(a)pyrene occurs preferentially on guanines adjacent to methylated cytosine. DNA repair is also thought to be more efficient in actively transcribed genes than in silent DNA regions, and to be faster on the DNA strand used as a template for transcription than on the coding strand. Thus strand bias in the distribution of mutations indicate, involvement of the exogenous carcinogens. However, experimental evidence for the role of transcription-coupled repair in such bias is still inconclusive (Hainut et al., 1998). Individual mutagens generate characteristic changes, which are known as fingerprints of DNA damage at specific locations along a given DNA sequence. The analysis of p53 mutation database has revealed such examples of patterns of mutations consistent with fingerprints of DNA damage induced by defined exogenous carcinogens. These examples include G→T transversion at codon 249 in hepatocellular carcinomas of patients with high dietary exposure to Aflatoxin B1 and tandem CC→TT mutations, a typical signature of UV-Induced mutagenesis. However, in most cancers, the patterns of mutations are complex. Cancers associated with tobacco smoking is a good example for this complexity. Smoking is a risk factor for several cancers, including cancers of the oral cavity, oesophagus, lung and bladder. In all pathologies, the prevalence of p53 mutations is generally higher in smokers than in non-smokers. However, the spectrum of p53 mutations varies from one pathology to the other. For example, the typical signature mutations by benzo(a)pyrene in lung cancers are G→T.
transversions. Experimental evidence shows that benzo(a)pyrene preferentially form
adducts at codons 157, 248 and 273, which are all mutation hot spots in lung cancer.
In squamous cell carcinoma of oral cavity and oesophagus, the predominant types of
mutations are transitions or transversions at A:T base pairs and G → A transitions. In
these cancers the combined consumption of tobacco and alcohol is considered to be a
cumulative risk factor and the observed spectrum is consistent with the role of N-
nitrosamines (G:C transitions) and of metabolites of ethanol such as acetaldehyde
(mutations at A:T bases) (Hainut et al., 1998). In principle, the p53 mutation spectrum
data can be used to generate hypotheses regarding disease risk factors in a defined
population (Hussain and Harris, 1999). In practice, however, interpretation of p53
mutation patterns observed in human tumours from murine models has proved
difficult. Although, there are yeast-based in-vitro methods to generate mutation
spectra in human p53 sequences there have been no practical in vivo approaches
(Fronza et al., 2000). The murine p53 gene is not an optimal target sequence to
explore origins and modulators of p53 mutation patterns found in human tissues,
because the mouse p53 gene differs from the human coding sequence at 15% of
residues. However, human p53 knock-in mouse (HUPKI) mouse was engineered to
replace core domain of the endogenous murine p53 allele with the homologous
normal human p53 gene sequence (Luo et al., 2000). Hence, this model offers an in
vivo approach for such investigations and comparison with human data. It is already
known that methylating agents such as MNNG and MNU have been shown to
produce gastric cancer (Preussmann et al., 1984) and dimethyhydrazine is associated
with colorectal cancer (Druckrey et al., 1967). These agents generate methylating
agents after metabolic activation and result in DNA adducts in target organs (Herron
and Shank, 1980; Preussmann and Stewart, 1984). The levels of O6-MedG adduct
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were also found to be increased in individuals with intragastinal tract tumours (Hall et al., 1991; Jackson et al., 1996; Krytopoulos et al., 1984; The Eurogast Study Group, 1994; Umbenhauer et al., 1985). All these data suggests that methylating agents may be involved in the etiology of these cancers. Therefore, intragastric nitrosation and intraintestinal nitrosation were thought to be major sources of alkylating agents. Further evidence for this hypothesis is the observation in human volunteers that increased protein intake results in elevated levels of intra-intestinal total NOC and a number of epidemiological studies have linked high protein intake with increased risk for GI tract, particularly colorectal cancers (UK department of health, 1998). However, it is unlikely that MNNG and MNU occur naturally in the GI tract indicating that major targets of nitrosation are amino acids and dietary proteins (Mirvish, 1995; Sheperd and Lutz, 1989). The nitrosation products of glycine would probably constitute a major source of alkylations agents, as glycine is one of the most abundant amino acids in nature (Sedgwick, 1997). It has already been reported that $O^6$-CMdG, the major $O^6$-guanine adduct of nitrosated glycine derivatives, is not repaired by $O^6$-alkylguanine-DNA-alkyltransferase, so could accumulate in the DNA of GI tissues as promutagenic lesion (Shuker and Margison, 1997). Further evidence of the likely human exposure of nitrosated glycine is based on the fact that $O^6$-CMdG is indeed detectable in gastric biopsies and human blood DNA samples using a sensitive immunoslot blot (ISB) assay (Cupid et al, 2004; Singh et al., 2000). Potassium diazoacetate (KDA) is particularly interesting because it is a stable nitrosated derivative of glycine which decomposes or rearrange to give carboxymethylating agents resulting in the formation of $O^6$-carboxymethyl-2'-deoxyguanosine and $O^6$-methyl-2'-deoxyguanosine. It was also reported that KDA is a powerful alkylating agent than APNG. It produced 8-fold increase in $O^6$-
carboxymethyl-2'-deoxyguanosine and 11 fold increase in \(O^6\)-methyl-2'-deoxyguanosine (Harrison et al., 1999). Although KDA was initially synthesised in 1908 its genetic effects have been never investigated. In the wake of the recent findings that linked high dietary protein consumption with colorectal cancers, it would be interesting to investigate the role of KDA via carboxymethylating adducts in colorectal cancers. The highly evolved genetically engineered Hupki mice with human p53 gene sequence means that we can generate KDA p53 gene mutation spectra experimentally. Thus, information regarding mutational specificity of KDA such as types, locations, hotspots and frequency of mutations can be obtained. This will allow us to compare the experimental p53 mutation data to human p53 mutation data of colorectal cancers from IARC database that will further help us to understand the mechanisms of mutation induction and carcinogenesis by nitrosoglycine derivatives. KDA was synthesised according to reported synthetic route (Kreevoy and Konasewich, 1970). The potassium diazoacetate was freeze-dried to obtain a fine yellowish powder and was characterised using LC-MS. \((M+H^+)\) found at 123.96 observed with positive ion mode confirmed that the product obtained was potassium diazoacetate. Two independent cytotoxicity tests were performed twice to determine optimal mutagenic dose \((IC_{50})\) of potassium diazoacetate by monitoring cell growth and calculating percentage viability of cells. The results of cytotoxicity tests showed us a trend of decreasing number of cells with increasing concentrations of KDA. Cell growth was monitored by counting the cells present in KDA incubated samples with respect to controls at regular intervals. Percentage viability was calculated by dividing the total number of cells in KDA treated sample by number of cells present in controls at that respective time point. The results of the cytotoxicity tests indicated that 10\(\mu\)M KDA dose is the optimal mutagenic dose \((IC_{50})\) to obtain mutant clones. Previous
cytotoxic studies on nitrosoglycine derivatives have reported to observe similar trend of decreasing percentage of viability on increasing dose. Previous studies on genotoxic effects of KDA were investigated by monitoring DNA damage in a comet assay. KDA showed dose related responses in the comet assay in vitro in the different cell types, over the dose range of 0 - 4nM (Anderson et al., 1999). Another study also reported dose dependent decrease in survival and mutation frequency in E.coli cells transfected with plasmid DNA treated with KDA over the dose range of 0 – 10mM (Gottschalg et al., 2006). Two independent mutagenesis experiments were carried out of which one experiment consisted of totally 48 clones exposed to 10μM of KDA and the other experiment consisted of 24 clones exposed to 100μM of KDA. The clones were passaged until mutant selection and clonal expansion process is completed. The fluorescent dideoxynucleotide cycle sequencing results of DNA binding domain (DBD) sequence of the p53 gene of seven of the clones treated with 10μM KDA did not show the presence of any functional mutations. The results suggested that KDA treated clones might have incurred spontaneous mutations by secondary mechanisms at some other down stream targets of p53 gene, which had lead to the formation of homogenous clones that survived the crisis period. Since, KDA is quiet unstable, it was assumed that as soon as KDA is mixed with DMEM media, it started decomposing. Therefore, the toxic effects of KDA to which cells were exposed might be low. Hence, further sequencing of the remaining clones was not carried out. Previous mutation studies of KDA on p53 gene using p53 yeast expression assay has indicated that most common mutations induced by KDA were base pair substitutions (Gottschalg et al., 2006). The mutations were obtained by treating plasmid, \(pLS76\) with KDA mixed either in 1X Tris-EDTA (TE) or Phosphate Buffered Saline (PBS Buffer). Approximately 57% of all mutations induced by KDA treatments in 1X TE
buffer were targeted at GC base pairs, where as 29% were directed against AT base pairs. Of these substitutions 53% were transitions, most of which were GC→AT (74%) base pairs. Transversions accounted for 47% of the base pair substitutions and were comprised largely of GC→TA (42%) and AT→TA (32%). The remaining mutations (14%) were identified to be mainly base pair deletions (Gottschalg et al., 2006). In contrast to treatments carried out in TE buffer, the percentage of mutations directed against AT base pairs is elevated to 43% with KDA treatment in 1X PBS buffer. 49% were transitions, most of which were GC→AT (74%) base pairs. Transversions accounted for 51% of the base pair substitutions and encompassed mainly AT→TA (32%) [Appendix 9] (Gottschalg et al., 2006). KDA mutation spectrum obtained from yeast-based p53 functional assay was compared with stomach p53 mutation spectra data from IARC database. One of the most common base pair substitutions in stomach cancer is the AT→GC transition at codon 220. KDA was shown to be able to induce this mutation in vitro. One of the gastric mutational hotspot, a GC transversion at codon 176 was also seen in the KDA- induced p53 spectrum in vitro. GC→AT mutations at codons 146, 151, 192, 277 and 278 were observed in the yeast based assay as with p53 database. However, other hotspots in the gastric tumour spectrum such as GC→AT transitions at codons 138, 159, 173 and 289 were not observed (Gottschalg et al., 2006). KDA was able to induce GC→AT transition at codon 241 that is an abundant hotspot in colon cancer according to IARC p53 database. Similar observations were made at codons 146, 238, 272 and 278. The statistical comparison of in vitro p53 mutation spectra data with IARC gastric p53 mutation data using Cariello’s software indicated that that the two spectra were different (Gottschalg et al., 2006). Hence, it would be more interesting to repeat this experiment with Hupki mouse model that has several advantages over other in vivo
and in vitro models. It will enable us to obtain information regarding the mutagenic specificity of KDA that is quite closer to the data of human p53 mutation spectrum that will enable better understanding of the mechanisms of mutagenesis by nitrosoglycine derivatives. DNA was extracted from HUF embryonic fibroblasts incubated with increasing KDA concentrations for adduct analysis. These samples were supposed to be analysed using immunoslot-blot (ISB) procedure to detect and measure the amount of $O^6$-alkylguanine adducts present in the KDA treated DNA samples. The idea was to observe a trend of increase in the number of adducts with an increase in the concentrations of KDA. Since the project has reached its final stage and so funding and time allotted to his project, this analysis has not been carried out.
Chapter 6: Conclusions and Future Work

6.0 Introduction

The carcinogenic activity of many chemicals is found to depend upon their ability to act as electrophiles. Some carcinogens are inherently electrophilic and some spontaneously hydrolyse to active species in solution or are activated by cellular enzymes. The resultant reactive intermediates modify the constituents of DNA, forming covalent adducts in which the carcinogen residue is joined to nucleophilic atoms of the constituent nucleotides. DNA adducts have the potential to force replication errors and repairs and thus be chemical progenitors of heritable genetic alterations. \(O^6\)-carboxymethyl-2'-deoxyguanosine is one of those DNA adducts, which has interesting features like \(O^6\)-methyl-2'-deoxyguanosine is also not repaired by repair enzyme \(O^6\)-alkylguanine-DNA-alkyltransferase. Several different aspects of DNA adduct, \(O^6\)-carboxymethyl-2'-deoxyguanosine have been investigated as a part of this project. Areas of research include synthesis and characterization of DNA adduct, \(O^6\)-carboxymethyl-2'-deoxyguanosine. Synthesis and characterization of oligonucleotides containing \(O^6\)-alkylguanine adducts using nucleoside composition analysis and fluorescence assays. Base-pairing stability of \(O^6\)-alkylguanine adducts were investigated using meting curve studies. The mutagenic properties of \(O^6\)-carboxymethyl-2'-deoxyguanosine adduct (\(O^6\)-CMdG) was investigated using ARMS PCR assay technique. Mutation spectrum of potassium diazoacetate was also investigated using mouse embryonic fibroblast cell lines as a part of this research project. The main findings and conclusions are summarised in the following sections, along with some indications of future directions.
6.1 Conclusions

6.1.1 Synthesis of $O^6$-carboxymethyl-2'-deoxyguanosine phosphoramidite

Chemical alkylation of DNA bases is believed to play an important role in the induction of mutagenesis and carcinogenesis. For example, formation of $O^6$-methylguanine and $O^4$-methylthymine has a causative role in carcinogenesis. Interestingly, bile acid derivative such as $N$-nitrosoglycocholic acid reacts with DNA to give rise to $O^6$-MedG and $O^6$-CMdG adducts (Shuker et al., 1997). The mutagenic potential of $O^6$-MedG adduct has already been well established. However, physical, chemical and biological properties of $O^6$-CMdG adduct have never been reported before. In addition, another interesting feature of $O^6$-CMdG adduct is that it is not repaired by repair enzyme $O^6$-alkylguanine-DNA-alkyltransferase. One of the central problems in the field of carcinogenesis was lack of an experimental system to identify which DNA lesion gave rise to which types of mutations. Hence, it is difficult to implicate the specificity and true mutagenic potential of $O^6$-carboxymethyl-2'-deoxyguanosine adduct with random mutagenesis studies. As a result, it is necessary to chemically synthesise $O^6$-carboxymethyl-2'-deoxyguanosine adduct and then incorporate site-specifically in to oligonucleotides to study physical, chemical and biological properties of this adduct in isolation. The reported method of synthesis is primarily based on the preparation of $O^6$-sulfonated derivative, which is quite reactive and can be easily displaced by amine and then alkoxide ions. $O^6$-carboxymethyl-2'-deoxyguanosine adduct is chemically synthesised by introducing alkyl group modification by displacing an arylsulphonate present group at $O^6$-position of 2'-deoxyguanosine. The reactive 3'-OH and 5'-OH groups of the 2'-deoxyguanosine were protected with acetyl groups instead of methoxyacetyl groups previously used for the protection (Harrison et al., 1997). The acetylation procedure used in the
reported method is a very slow reaction. However, when catalyst
dimethylaminopyridine was used, it hastened the process much faster than original
method. It has been documented that acyl groups at $N^2$- position of $O^6$-substituted
guanines are much more resistant to removal by ammonia than those at $O^6$-
unmodified guanine. Therefore, a more base-labile group such as phenylacetyl group
was chosen to protect exocyclic $N^2$-position of guanine instead of isobutyryl group.
This allowed selective removal of acetyl groups at 3' and 5'-OH position with out
removing phenylacetyl group at $N^2$-position of guanine. Reagent phenylacetyl
chloride, which was used for this reaction is highly reactive forming many side
products. This caused not only the process of purifying desired compound difficult
but also reduced the yield of the product. Better yields and purity can be achieved
using phenylacetic anhydride reagent. However, the highly unstable characteristic of
phenylacetic anhydride is a limiting factor. The 3',5'-diacetyl-N2-phenylacetyl-2'-
deoxyguanosine was first converted in to arylsulphonate derivative, 3',5'-diacetyl-N2-
phenylacetyl-6-mesitylenesulphonyl-2'-deoxyguanosine. The arylsulphonate
derivative is very sensitive and it was found that any attempts to purify this compound
on silica column cause degradation of the compound. Hence, it was straight away
converted in to quinuclidinium salt, which in turn was replaced by methylglycolate
moiety. The acetyl groups protecting the hydroxyl groups of the sugar were
selectively removed using triethylamine in methanol instead of using aqueous NaOH.
This is because the later procedure is less specific and there is always a high risk of
losing N2-phenylacetyl protecting group. However, it was observed that often it is not
possible to crystallise deacetylated product from methanol. Addition of 4',4'-
dimethoxytrityl group to 5'-OH group and its purification is a bit tricky process. In our
findings, it was observed that after certain time point, equilibrium of the reaction do
not shift in either directions. When the reaction was monitored on TLC, it appears that
the amount of the starting material is equivalent in proportion to the final product. The
equilibrium of reaction does not shift towards forward direction even after adding
increasing amounts of DMT-chloride. Since, silica has acidic properties and DMT
group is vulnerable to hydrolysis in acidic environment, basic conditions were
maintained by adding few drops of pyridine to every fraction of eluting solvent to
purify the compound. Dimethoxytritylation of the 5'-OH to give compound 6 was
accomplished in acceptable yield (65% after chromatography). Finally, reaction with
2-cyanoethyl- $N$, $N$-diisopropyl chlorophosphoramidite for 30 min at ambient
temperature followed by a methanol quench gave the desired $O^6$ phosphoramidite 7 in
46% yield after chromatography. Chemical characterisation data, final yields and
purity of all intermediates and final compound is in accordance with earlier reported
data.

6.1.2 Automated Synthesis of Oligonucleotides containing $O^6$-alkylguanine
Adducts

Synthetically modified nucleic acids can serve as powerful probes for studies on types
of confirmations assumed my modified adducts in helix structure of DNA, protein-
nucleic acid interactions and of course to study the biological properties of a DNA
adduct in a cellular environment. Modified synthetic phosphoramidites are
particularly useful, since they allow site-specific incorporation of modifications in to
DNA or RNA oligonucleotides. Automatic DNA synthesis is based on the principle of
solid phase synthesis. Heterogeneous reactions involved in the DNA synthesis are
standardised, and these procedures were easily automated. $\beta$-cyanoethyl
phosphoramidite chemistry is chosen for internal incorporation of phosphoramidite
analogue in to a series of oligonucleotides using automated DNA synthesis. The conditions of oligonucleotide synthesis via phosphoramidite chemistry had to be adjusted to take in to account the altered structure and reactivity of monomers compared to standard phosphoramidites. As part of this project, a method is developed particularly for convenient synthesis of oligonucleotides containing $O^6$-alkylguanine phosphoramidites. It was observed that using this method, modified $O^6$-alkylguanine adducts can be efficiently incorporated in to a DNA sequence. The method developed to synthesise modified oligonucleotides is called as “Push” and “Pull” method, which involves interruption of automated DNA synthesis at adduct site and addition of modified DNA adducts on to support manually. The advantage of this method is that it uses less amount of modified adduct to synthesise modified oligonucleotides at reasonable yields. Significant differences in overall synthetic yields were not observed between oligonucleotides made with normal deoxynucleotide phosphoramidites and those which containing the analogues ($O^6$-MedG and $O^6$-CMdG) indicating that the modified $O^6$-alkylguanine phosphoramidite is a suitable reagent for automated oligonucleotide synthesis. Normal oligonucleotides were finally deprotected by treating with ammonium hydroxide solution overnight at room temperature followed by incubation at 55° C for 2hrs. The similar conditions if used for deprotection of oligonucleotides containing $O^6$-MedG adduct causes not only stabilisation of the exocyclic amino protecting $N^2$-isobutyryl group of $O^6$-MedG, but also $O^6$-alkyl groups become vulnerable to displacement by ammonia leading to the production of oligomers containing 2',6'-diaminopurine. Therefore, oligonucleotides containing $O^6$-MedG adducts were deprotected by treating with 10% DBU in methanol solution and left in dark for 5days. It was already reported that $O^6$-CMdG adduct when deprotected with ammonia produced mainly an oligonucleotide
containing amide at $O^6$ position of guanine. Hence, these oligomers were deprotected by treating with 0.5M NaOH solution overnight. This method of deprotection will only just hydrolyse the ester group at $O^6$-position of guanine and will not remove the $O^6$-carboxymethyl group. This was explained by the fact that the alkaline solution initially hydrolyses the ester group and the resultant negatively charged carboxylate anion prevents further nucleophilic attack by hydroxide ion on the $O^6$-position of the guanine. All synthetic oligonucleotides are subjected to some level of contamination depending upon the method of preparation. The purity requirement is not very stringent for oligonucleotides used for structural determination studies. However, if the oligonucleotides were to be used for genetic studies, the levels of tolerable impurities are very low. Hence the oligonucleotide that were used in our studies are initially purified with Nensorb Prep cartridges to remove all reaction intermediates salts and incomplete short sequences. These are further purified using fast protein liquid chromatography (FPLC). All the oligonucleotide sequences were analysed on both HPLC and FPLC and found that they are in excess of 98% pure and contained modified $O^6$-alkylguanine adducts.

6.1.3 Characterization of Oligonucleotides using Nucleoside Composition Analysis and Fluorescence Assays

It is very important to characterise oligonucleotides to verify the presence of modified adducts. It is particularly important because, it is necessary to determine that alkylation modification introduced at $O^6$ position of 2'-deoxyguanosine is stable to the conditions of automated DNA synthesis, deprotection, purification conditions of FPLC and of course to avoid false positive results from adduct specific mutagenesis studies. This analysis is an enzymatic reaction in which the pentamers were
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hydrolysed to individual nucleosides, which will be monitored on HPLC using either UV or fluorescence detector. Since, pentamers are short sequences, it is easy to detect signal from modified base without any disturbance from background produced by normal nucleosides. Initially, all the pentamers were characterised on FPLC by observing the individual retention times. From the results of FPLC analysis on pentamers, it was confirmed that all three pentamers have different retention times. The results were confirmed not only by observing the differences in retention times for each pentamer separately but also by co-injection of all three pentamers together. Additionally, it was also confirmed that modified oligonucleotides were eluting later than normal pentamer due to presence of extra negative charge on $O^6$-alkylguanine adducts. The enzymatic degradation of both normal and modified pentamers provided the correct ratios of unmodified and modified nucleosides. Chemically synthesised $O^6$-MedG and $O^6$-CMdG nucleosides were used as standards. Both the chemically synthesised and enzymatically obtained $O^6$-MedG and $O^6$-CMdG nucleosides were confirmed to have similar retention times from the nucleoside composition analysis results. The UV spectrum of both $O^6$-MedG and $O^6$-CMdG nucleosides have $\lambda_{\text{max}}$ at 247, which confirms the fact that both $O^6$-MedG and $O^6$-CMdG nucleosides were made from starting material 2'-deoxyguanosine and $\lambda_{\text{max}}$ 280 is associated with the alkyl modification introduction at $O^6$ position of 2'-deoxyguanosine. The main drawback of this assay is that it cannot be applied effectively on longer oligonucleotides that were used for adduct specific mutagenesis studies. It is practically difficult, if not impossible to detect the presence of modified nucleoside from normal nucleosides due to strong background. However, this technique is utilised to confirm the presence of modified adducts in longer oligonucleotides by taking advantage of the fact that $O^6$-alkylguanines are slightly more fluorescent than
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the corresponding deoxynucleosides. Single peak was observed for all the sequences when monitored on HPLC confirmed that all sequences used for adduct specific mutagenesis studies were in excess of 98% pure. The modified nucleosides from enzymatic reaction were monitored using fluorescence detector instead of UV detector. The enzymatically obtained modified nucleosides at excitation wavelength 286nm and emission wavelength 378nm were observed to have similar retention times as chemically synthesised standards. Additionally when co-monitored with UV detector, the fluorescent peaks observed for $O^6$-MedG and $O^6$-CMdG nucleosides obtained from enzymatic digestion were observed to have similar UV absorption profile ($\lambda_{max}$ at 247nm and 280nm) as their chemical standards.

6.1.4 Melting curve analysis on Self-Complementary Oligomers containing $O^6$-Alkylguanine Adducts

Our Melting curve studies fairly indicated that $O^6$ – alkylguanine adducts if formed, would effect base pairing property and probably induce mutations. Qualitatively, the differences in base pair stabilities appear to be sufficient. The melting curves obtained were not ideal S-shaped curves. The midpoint of the raising portion of these S -shaped curves defines the melting temperature $T_m$, a rough indicator of the stability of the oligonucleotide complexes. Hence, exact melting temperatures were not determined. However, the data obtained from melting temperature profiles and the first derivative curves of melting profiles have given estimate about the thermodynamic properties and stabilities of base pairs of $O^6$ – MedG, and $O^6$ – CMdG adducts. The $O^6$ – alkylguanine adducts were incorporated in to self-complementary sequences of the types 5' CGC XAG CTT GCG 3' and 5' CGC XAG CTC GCG 3' at position X. The melting curve profiles and derivative curve data confirmed that
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introduction of G:T mismatch pair in normal sequence 4 caused reduction in melting temperature causing destabilisation effect on hairpin structure, with respect to normal sequence 5 containing G:C base-pair. Our studies have also confirmed that O\textsuperscript{6}-MedG:T base pair formed in sequence 6 is relatively much more stable than O\textsuperscript{6}-MedG:C base pair formed in sequence 7. O\textsuperscript{6}-MedG:T base pair has induced increase in melting temperature with respect to normal sequence 4 containing G:T mismatch base pair. In other words, it was understood that O\textsuperscript{6}-MedG:T base pair caused little destabilisation of hairpin structure than it could be caused by introducing G:T mismatch base pair. In addition, it was also observed that sequence 7 containing O\textsuperscript{6}-MedG:C base pair caused marginal reduction in melting temperature with respect to normal sequence 5 containing G:C base pair. However, O\textsuperscript{6}-MedG:C base pair containing sequence 7 denaturises earlier than O\textsuperscript{6}-MedG:T base pair containing sequence 6 suggesting that the later is much more stable base pair. In addition the melting temperature at the point of maximum absorption is estimated to be in the range 70 - 80°C for sequence 6 containing O\textsuperscript{6}-MedG:T base pair, whereas it is around 60 °C, for sequence 7 containing O\textsuperscript{6}-MedG:C base pair. The exact melting temperatures for self-complementary sequences containing O\textsuperscript{6}-CMdG adducts were not determined due to technical constraints of the instrumentation. Sequence 8 and 9 containing O\textsuperscript{6}-CMdG:T and O\textsuperscript{6}-CMdG:C base pairs induced increase in melting temperatures with respect to normal sequences 4 and 5 containing G:T and G:C base pairs. It is understood from the above data that O\textsuperscript{6}-CMdG:T base pair do not have strong destabilising effect on the hairpin with respect to sequence 4 containing mismatch base pair G:T. In addition, the data from derivative curve indicated that sequence 8 containing O\textsuperscript{6}-CMdG:T base pair denaturises later than sequence 9 containing O\textsuperscript{6}-CMdG:C base pair. This suggests that O\textsuperscript{6}-CMdG:T base pair might be
having less destabilising effect on hairpin structure indicating that $O^6$-CMdG adduct might preferentially base pair with T, thereby inducing GC $\rightarrow$ AT transition mutations. Clearly further experimentation and effort is needed to substantiate the validity of the above data.

6.1.5 Characterization of Mutations Induced by $O^6$-alkylguanine adducts

Due to the diversity of adducts produced by certain mutagens, it is desirable to relate the mutations produced to the structure and position of a given lesion. In conducting such experiments, it is essential to establish the degree of homogeneity and purity of the mutagenic species so that experimental results are not confounded by the presence of minor components. For example, previous mutagenesis studies on KDA, a carcinogen of our particular interest that predominantly forms $O^6$-CMdG adduct, was found to induce mainly GC – AT and other transition mutations in p53 cDNA. It is also known that KDA reacted with DNA to form $O^6$-MedG adduct. Hence, it is practically difficult, if not impossible to determine which adduct causes which mutation and why. Hence as a part of this research project, a site-specific plasmid based mutagenesis approach was designed, in which a single DNA adduct was uniquely placed in either strand of plasmid vector to study the genotoxic effects of the DNA adduct, $O^6$-carboxymethyl-2'-deoxyguanosine. $O^6$-CMdG adduct was synthesised according to reported synthetic route (Xu, 2000). The complementary oligonucleotide sequences 25 and 26mer used to form adduct containing duplex were designed from the p53 cDNA sequence of E.coli plasmid, pLS76. $O^6$-alkylguanine adducts were incorporated in to codon 196 of p53 gene at nucleotide position 587 for duplexes containing adduct on sense strand and at nucleotide position 586 for duplexes containing adduct on anti-sense strand. The normal and adduct containing
duplexes were successfully prepared by annealing complementary sequences 25 and 26mer. The duplexes were purified from unannealed single strands on nondenaturing polyacrylamide gel, removing all other impurities to construct a pure adduct containing plasmid. However, the restriction site BbvC1, formed at 5'-end by these duplexes with which they ligate with gapped plasmid was found to have poor religation efficiency. In addition, other restriction site Apol, formed at 3'-end by the duplexes was found to be a unique restriction site in p53 cDNA, but not in whole plasmid DNA sequence. Nevertheless, a brilliant strategy was designed overcoming these limitations to construct the intact plasmids containing O6-alkylguanine adducts. The duplexes were successfully incorporated in to E.coli based plasmid, pLS76 to form a double stranded DNA shuttle vector containing either a single O6-MedG or O6-CMdG adduct. Plasmids incorporated with adducts were characterised by PCR reaction. PCR results showed that all plasmids incorporated with O6-alkylguanine adducts when amplified with p53 specific primers produced 800bp p53 cDNA. These results indicated that adduct containing duplex DNA was efficiently incorporated to form intact adduct containing shuttle vectors. The advantage of studying the mutations induced by a lesion in double stranded vector is that the system comes as close as possible to resembling genomic replication. Amplification Refractory Mutation System (ARMS) technique was used to study the type of mutations that can be induced by O6-alkylguanine adducts. This technique is designed to discriminate between target DNA sequences that differ by a single nucleotide in the region of interest, codon 196. Complementary primers that amplify the region of interest were site specifically designed to differ at the nucleotide that occurs at the extreme 3' terminus. Since the DNA synthesis step in a PCR reaction is dependent on correct base pairing at 3'-end, this method is particularly useful in detecting any pathogenic
mutations. This principle behind this technique is similar to primer extension reaction except that nucleotide that is going to base pair with DNA adduct is already present at the 3' terminus of primer. The results of ARMS-PCR assays have indicated that O6-CMdG adduct is substantially mutagenic and force translesion synthesis in *in vitro* system. PCR assay have shown that positive control plasmids have normal GC base pair present at adducted site i.e., nucleotide position 587. The results obtained from plasmid pLS76 - 2 containing O6-MedG adduct were different from plasmid, pLS76 - 4 containing O6-CMdG adduct. When both plasmids, pLS76 - 2 and pLS76 - 4 containing adduct on sense strand at nucleotide position 587 in codon 196 were compared, O6-MedG adduct was preferably inducing GC - AT transition and O6-CMdG adduct induced GC - TA transversion mutation. However, results obtained from plasmids, pLS76 - 3 and pLS76 - 5 containing adducts on anti-sense strand i.e., at nucleotide position 586 in codon 196 indicated that both O6-MedG and O6-CMdG adducts induced GC – TA transition mutation. The results from positive control plasmids confirmed the presence of normal GC base pair at nucleotide position 586 on anti-sense strand. Nevertheless, of its location on either strand, O6-MedG adduct predominantly induced GC-AT transition mutations. The results supports the following explanations offered to support the true mutagenic potential of O6-MedG adduct. Firstly, DNA polymerase might mistake O6-methylguanine for adenine because of the physical similarity of these bases. Both, O6-methylguanine and adenine are similarly lipophilic and the X-ray crystallographic studies on the nucleosides have shown a close similarity in bond angles and lengths between O6-methylguanine and adenine. Secondly, the important factor in the miscoding is that the alkylG.T mismatch pair retains the Watson-Crick alignment with N1 of the purine juxtaposed to N3 of the pyrimidine. However, results obtained from O6-CMdG adduct were quiet
surprising. Because the $O^6$-CMdG is a guanine adduct modified at $O^6$ position just like $O^6$-MedG, was expected to induce GC-AT transitions preferably. Nevertheless, it has induced GC-TA transversion mutations when present on sense strand and GC-AT transition mutations when present on anti-sense strand. The qualitative data has clearly indicated that $O^6$-CMdG adduct behaves more like a bulky adduct that can induce multiple mutations. However, quantitative data relating to mutation frequency, specificity, and relative effects of the DNA strand and flanking sequences on mutagenic potential have to be determined to further substantiate the reliability of the above data. Further experimentation and effort is also needed to probe the mutagenic mechanisms of $O^6$-CMdG adduct in in vivo systems.

6.1.6 Characterisation of KDA p53 Mutation Spectra using Hupki mouse model

The Promutagenic potential of potassium diazoacetate is studied as a part of a collaborative project with Dr Monica Hollstein in German Cancer Research Centre, Heidelberg, Germany. Human p53 knock-in mouse (HUPKI) mouse in which core domain of the endogenous murine p53 allele has been replaced with the homologous normal human p53 gene sequence was treated with KDA to obtain mutant clones. The aim of this collaboration was to compare in vivo mutation spectrum with the p53 mutation database at IARC and possibly identify KDA as risk factor in certain type of malignancies. Potassium diazoacetate (KDA) is particularly interesting because it is a stable nitrosated derivative of glycine. KDA will decompose or rearrange to give carboxymethylating agents resulting in the formation of $O^6$-carboxymethyl-2’-deoxyguanosine ($O^6$-CMdG) and $O^6$-methyl-2’-deoxyguanosine ($O^6$-MedG) adducts. KDA was synthesised according to reported synthetic route and characterised on LC-MS. Cytotoxicity tests were performed twice and the optimal mutagenic dose ($IC_{50}$)
of potassium diazoacetate was calculated from cell growth and percentage viability of cells. A trend of decreasing number of cells in samples incubated with increasing concentrations of KDA was observed. Cell growth was monitored by cell counting at regular intervals and percentage viability of cells was calculated by dividing the number of cells present in samples treated with KDA with number of cells present in respective controls. Two independent mutagenesis experiments were carried out of which one contain 48 clones incubated with 10μM of KDA and the other contained 24 clones treated with 100μM of KDA. Clones were passaged until selection process and clonal expansion is completed. The results of the sequencing of p53 gene of some of the clones obtained by treating with 10μM KDA indicated that KDA did not induce any functional mutations in the DNA binding region of p53 gene (exon 4 – 9). The results have indicated that KDA might have decomposed on mixing with DMEM, therefore subdued the toxic effects of the KDA to which cells were exposed. The results also suggested that spontaneous mutations by secondary mechanisms at some other down stream targets of p53 gene might be responsible for the formation of homogenous clones that survived the crisis period. Hence, further sequencing of the remaining clones was not carried out. Since time and funding allotted to this project have reached to their limitations, DNA adduct analysis using ISB technique was not carried out.

6.1.6.1 Further Mutagenesis Studies using p53 Yeast Expression System and Hupki Mouse

Endogenous nitrosation of dietary amino acids and peptides has been proposed as a major route of exposure to genotoxic agents in the gastrointestinal tract (Challis, 1989; Shephard, 1989). Cupid et al. (Cupid, 2001) showed that diazoacetate was
formed upon treatment of glycine with NO at conditions similar to those found in the GI tract \textit{in vivo}. Moreover, incubation of the reaction mixture with 2.-dG and DNA resulted in the formation of $O^6$-CMdG and $O^6$-MedG DNA adducts. These findings suggested that diazoacetate was a key alkylating agent formed from the nitrosation of glycine under simulated physiological conditions. Potassium diazoacetate (KDA) is particularly interesting because it is a stable nitrosated derivative of glycine which decomposes or rearrange to give carboxymethylating agents resulting in the formation of $O^6$-carboxymethyl-2'-deoxyguanosine and $O^6$-methyl-2'-deoxyguanosine. It was also reported that KDA is a powerful alkylating agent than APNG. It produced 8-fold increase in $O^6$-carboxymethyl-2'-deoxyguanosine and 11 fold increase in $O^6$-methyl-2'-deoxyguanosine (Harrison et al., 1999). $O^6$-CMdG has also been detected in human gastric biopsies (Singh, 2000). Previous mutagenesis studies on KDA have reported that this compound induced some p53 mutations found in colon and stomach cancer. Hotspots such as the AT$\rightarrow$GC transition at codon 220 were found in both human spectra and the \textit{in vitro} spectrum. One of the major hotspots found in colon cancer, a GC$\rightarrow$AT transition at codon 241 was also common to the KDA-induced p53 spectrum (Gottschalg et al., 2006). Our experiments on KDA to study its mutation spectra in p53 gene using murine model HUPKI mouse did not showed the presence of any functional mutations. However, this is mostly due to the stability problems associated with KDA rather than the system. Surely better results can be expected with this, if we overcome the problem associated with KDA stability. The mutation spectrum data obtained for KDA from p53 yeast expression system means that similar data can be obtained from the above murine model. Also, as a part of this research project we have successfully synthesised $O^6$-CMdG adduct and could incorporate it site specifically in to p53 cDNA. Although, our results obtained with ARMS-PCR
assay technique which used this site specific sequences indicated that \( O^6\text{-CMdG} \) adduct is qualitatively mutagenic. However, it would be more interesting to see whether \( O^6\text{-CMdG} \) adduct can induce similar mutations in vivo system such as p53 yeast expression system. This system could not only verify our data qualitatively but also can produce quantitative data relating to mutation specificity and frequency. Alternatively, incubation of KDA-treated yeast expression vector pLS76 using \( O^6\text{-alkylguanine-DNA alkyltransferases} \) prior to transformation into yeast cells might allow the study of \( O^6\text{-CMdG} \) specific mutagenesis. As this adduct was shown to be not repaired by \( O^6\text{-alkylguanine-DNA alkyltransferases} \) (Shuker, 1997) it might not only be an important and possibly persistent biomarker but also a contributor to some types of human malignancies.
6.2 Overall Conclusions

The main objectives set out at the beginning of this project could be achieved in the majority of cases. $O^6$-carboxymethyl-2'-deoxyguanosine adduct was successfully synthesised and incorporated site specifically into p53 sequences. The results from nucleoside composition analysis had confirmed that these site specifically modified sequences contain $O^6$-CMdG adduct. Melting curve studies have shown qualitative differences in the stabilities of base pairs formed by $O^6$-CMdG adduct with respect to controls containing $O^6$-MedG adduct and 2'-dG. However, quantitative data was not obtained from the above meting curve studies due to the technical limitations of the instrumentation. Thus future studies need to focus on obtaining quantitative differences in the stabilities of the base pairs.

The important aspect relating to the mutagenic potential of $O^6$-CMdG adduct had been investigated using Amplification Refractory Mutation Sytem (ARMS) PCR technique. Qualitative data from the above studies have suggested that $O^6$-CMdG adduct is a potential mutagenic adduct and can induce multiple mutations like a bulky adduct. However, further studies are needed to investigate the mutagenic specificity and frequency of the $O^6$-CMdG adduct.

Results of the mutation spectrum studies on potassium diazoacetate, a stable nitrosoglycine derivative indicated that the above compound did not induce any functional mutations in the p53 gene of HUPKI mouse embryonic fibroblast cells. However, this is mainly due to problems associated with KDA stability. Previous studies on KDA mutagenic potential which used p53 yeast expression system have reported that this compound induced some p53 mutations found in colon and stomach cancer. Hence, further effort and experimentation is needed to overcome the problems.
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associated with KDA stability to study the mutation spectrum of KDA using HUPKI mouse.
Chapter 7: Bibliography

7. Bibliography


risk of cancer of the oral cavity, oesophagus, stomach and urinary bladder. *Cancer Surv*, 8, 423-442.


8 Thesis Related Conferences

Publications


Conference Abstracts


- **Ponnada, S., Burns, P.A., Xu, Y-Z. and Shuker, D.E.G.** Characterisation and Significance of Mutations in p53 induced by Diazoacetate – A Possible Etiological agent for Gastric cancer. Presenting at NCRI Cancer Conference, 2-5 October 2005, International Conventional Centre, Birmingham, UK.
Appendix 1:

Proton Chemical Shift Values of Compounds 1-6:

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Compound 7 is characterised using $^{31}$P NMR

$^{31}$P NMR chemical shifts from compound 7 were observed at 151.15 and 151.20 using CDCl₃ as solvent. The two peaks represent two stereo isomers of the phosphoramidite monomer.
Appendix 2:

Characterisation of compounds 1-7 using LC-MC Mass spectrometer:

**Compound -1:**

MS m/z (positive ion mode)
[MH$^+$] - 351.32
[MH$^+$ - Sugar Fragment] - 151.40

**Compound-2:**

MS m/z (positive ion mode)
[MH$^+$] - 470.40
[MH$^+$ - Sugar Fragment] - 269.56
[MH$^+$ - Sugar Fragment - PAC] - 151.53

**Compound 3** - It is an unstable compound and undergoes hydrolysis very fast. Hence, it was not characterised using LC-MS.

**Compound 4:**

MS m/z (positive ion mode)
[MH$^+$] - 542.44
[MH$^+$ - Sugar Fragment] - 342.24
[MH$^+$ - Sugar Fragment - PAC] - 224.03

**Compound 5:**

MS m/z (positive ion mode)
[MH$^+$] - 458.30
[MH$^+$ - Sugar Fragment] - 342.61
[MH$^+$ - Sugar Fragment - PAC] - 224.05

**Compound 6:**

MS m/z (positive ion mode)
[MH$^+$] - 759.54
[MH$^+$ - DMT] - 302.24

All the LC-MS data was obtained using 50/50 acetonitrile/ammonium acetate (pH-5.4) solvent at the flow rate 0.2ml/min.
Appendix 3:

p53 cDNA Sequence:

```
1  ATG  GAG  GAG  CCG  CAG  TCA  GAT  CCT  AGC  GTC  GAG  CCC  CCT  CTG  AGT  15
16  CAG  GAA  ACA  TTT  TCA  GAC  CTA  TGG  AAA  CTA  CTT  CCT  GAA  AAC  AAC  30
31  GTT  CTG  TCC  CCC  TTG  CCG  TCC  CAA  GCA  ATG  GAT  GAT  TTC  AGT  CTG  45
46  TCC  CCG  GAC  GAT  ATT  GAA  CAA  TGG  TTC  ACT  GAA  GAC  CCA  GGT  CCA  60
61  GAT  GAA  GCT  CCC  AGA  ATG  CCA  GAG  GCT  GCT  CCC  CGC  GTG  GCC  CCT  75
76  GGA  CCA  GCA  GCT  CCT  ACA  CCG  GCC  GCC  CTC  GCA  CCA  GCC  GCC  TCC  90
91  TGG  CCC  CTG  TCA  TCT  TTC  GTC  GCT  TCC  CAG  AAA  ACC  TAC  CGG  GCC  105
106  AGC  TAC  GGT  TAT  CTG  CTG  GCC  GAG  AGG  AAT  TGT  TAC  TCT  TCT  GAT  120
121  TCT  GTG  ACT  TGC  AGC  TAC  TGC  CTC  CTT  GCA  ATT  TCT  AGG  CTC  GCA  135
136  CAA  CTG  GCC  AAG  ACC  TGG  GTC  GAT  GGA  CCA  GGT  GAC  AAG  TTC  AAC  150
151  CCC  CCG  CCG  GCC  GGC  ACC  CGC  CTC  CGC  GAC  GAT  ATT  GCT  AAC  ACG  165
166  TCA  CAG  CAT  TTG  GCAG  GAG  GGT  TGA  TCT  CGA  TAT  TGG  GTC  ACC  GAC  180
181  GCC  TGC  TCA  TCA  ATG  GAT  ACG  ATG  GCT  GAA  TTC  GCT  GCA  GGT  AGT  195
196  CAA  CTG  GAA  AAA  GAG  CTA  GGG  CAG  GCC  AGT  TCT  GGA  GCT  GCC  TGC  210
211  ACT  CTT  TTA  CGA  CAT  ATG  GAT  GTC  GGT  CCC  TAT  CAG  TAC  GGT  TAC  225
226  GCC  TCT  GAC  TGT  ACC  ACC  ATC  AAC  TAC  AGC  CCA  TGG  CCT  AAC  AGC  240
241  TCC  TGC  ATG  GCC  GCC  AAA  ATG  AAG  CCC  ATC  CTC  ACC  ATC  ACC  AAC  255
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346  GAC  GCC  TTG  GAA  ATG  GAT  GCC  GCT  GAA  AAC  GAG  CCA  CCC  TGT  GGA  360
361  GGG  AGC  AGG  GTC  CAC  TCC  AGC  CAC  CTG  AAG  TCC  AAA  AAG  GTG  CAG  375
376  TCT  ACC  TCC  CGC  CAT  AAA  AAA  CTC  ATG  TTC  AAG  ACA  GAA  GCC  CAC  390
391  GAC  TCA  GAC  TGA
```
### Appendix 4:

**p53 Mutation spectra data obtained from various murine model studies [Zielinski (2002):**

<table>
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<tr>
<th>Target Organ/ Tumour type</th>
<th>Treatment</th>
<th>Strain Description</th>
<th>Number of Lesions/ Number of Mice</th>
<th>Number of p53 mutated lesions/ Number of lesions Analysed (%)</th>
<th>Reference</th>
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<td>UVB</td>
<td>CBaxC57BL6xCD1, XPA-</td>
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<td>Takeuchi et al., (1998)</td>
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<td>42/14</td>
<td>6/42 (14)</td>
<td>Van Kranen et.al., (1997)</td>
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<td>DMBA/TPA</td>
<td>CD1</td>
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<td>45/10</td>
<td>0/n.g.</td>
<td>Shibita et al., (1997)</td>
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<td></td>
<td>MT4</td>
<td></td>
<td>72/9</td>
<td>0/n.g.</td>
<td>Shibita et al., (1997)</td>
</tr>
<tr>
<td>Lung</td>
<td>AFB1</td>
<td>AC3F1(A/J×C3H/HeJ)</td>
<td>76/62</td>
<td>20/76 (26)</td>
<td>Tam et al., (1999)</td>
</tr>
<tr>
<td></td>
<td>Coal Tar</td>
<td>B6C3F1</td>
<td>15/n.g.</td>
<td>0/15 (0)</td>
<td>Culp et al., (2000)</td>
</tr>
<tr>
<td>Liver</td>
<td>Coal Tar</td>
<td>B6C3F1</td>
<td>37/n.g.</td>
<td>4/37 (11)</td>
<td>Culp et al., (2000)</td>
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<tr>
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<td>AFB1</td>
<td>C57BL/6</td>
<td>08/33</td>
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<tr>
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<td>C3H</td>
<td>7/10</td>
<td>0/7 (0)</td>
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<td>14/16</td>
<td>0/9 (0)</td>
<td>Masui et al., (1997)</td>
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<td>B(a)P</td>
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<td>3/14 (10)</td>
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<td>B6C3 F1</td>
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<td>8/14 (57)</td>
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<td>C57BL6 (p53 +/-)</td>
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<td>Ozaki et al., (1998)</td>
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<tr>
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<td></td>
<td>C57BL/6</td>
<td>n.g./52</td>
<td>3/11 (27)</td>
<td>Ozaki et al., (1998)</td>
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<td>Strain</td>
<td>Number of Lesions/ Number of Mice</td>
<td>Number of p53 mutated lesions/ Number of lesions Analysed (%)</td>
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<tr>
<td>Uterine</td>
<td>ENU</td>
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<td>5/22 (23)</td>
<td>Culp et al., (2000)</td>
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<tr>
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<td>7/40 (18)</td>
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<td>Hong et al., (2000)</td>
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<td>0/8</td>
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n.g. – not given
## Appendix 5: Prevalence and Spectra of p53 mutations in Human Tumours

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<td>2</td>
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260
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<th>Prev. p53 Mut. c%</th>
<th>G:C→A:T %</th>
<th>G:C→TA %</th>
<th>G:C→C:G %</th>
<th>A:T→G:C %</th>
<th>A:T→T:A %</th>
<th>A:T→C:G %</th>
<th>Del/Ins/other %</th>
<th>CpG HOTSPOTS f</th>
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<tr>
<td></td>
<td>(n)</td>
<td>%</td>
<td>%</td>
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<td>%</td>
<td>%</td>
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</table>

- Mutation spectra of tumour type was calculated only if >20 mutations were reported.
- n – the number of tumours of each cell type evaluated for p53 mutation by PCR-based technique as compiled in the p53 mutation database.
- c – Prevalence of p53 mutations in cancers from each organ/tissue, as detected PCR techniques. Screening at least exons 5 – 8.
- del – Deletions
- Ins – Insertions
- e – % of mutations which are G:C→A:T transitions at CpG dinucleotides.
- f – Hotspots are defined as codons at which at least 50 mutations have been reported
- * – CpG Site
Appendix 6:

Prevalence of p53 mutations in different Tumour types

Prevalence of p53 Germ-line mutations in different Tumour types

(C) IARC TP53 Mutation Database, R10 release, July 2005
Appendix 7:

Somatic Mutations – Mutation Effect / 21587 Mutations

Somatic Mutations – Mutations Pattern / 21587 Mutations

(C) IARC TP53 Mutation Database, R10 release, July 2005
Appendix 8:

Germ-line Mutations – Mutations Effect / 283 Mutations

(C) IARC TP53 Mutation Database, R10 release, July 2005

Germ-line Mutations – Mutations Pattern / 283 Mutations

(C) IARC TP53 Mutation Database, R10 release, July 2005
Appendix 9:

Comparison of classes of between p53 mutations in human colon and stomach tumours. Data presented in above figure were adapted from p53 IARC database. GC to AT at CpG sites were not included [Elke Gottschalg nee Griech (2002)].

Comparison of classes of between p53 mutations induced by KDA treatments in 1x TE and PBS Buffer [Elke Gottschalg nee Griech (2002)].