Studies on nucleotide oxidation and glycation adducts in Type-2 Diabetes, ESRD and carbonyl induced glycation damage in some tumour cells
5.1 INTRODUCTION

The role of AGEs in the pathophysiological complications of diabetes is now well recognized (Sampathkumar et al., 2005). The AGEs are chemically heterogeneous and induce several cellular responses through interaction with AGE specific cell-surface receptors (Vlassara et al., 1988 and 1994; Esposito et al., 1989; Kirstein et al., 1990; Kirstein et al., 1992; Bucala et al., 1991). Direct evidence on the in vivo pathogenic effects of AGEs, independent of hyperglycemia was provided by the experiments in which intravenous administration of exogenous AGEs to healthy animals induced multiple vascular defects including vascular permeability, subendothelial and perivascular mononuclear cell infiltration, and defective nitric oxide-dependent vasodilatory responses (Makita et al., 1995). Accumulation of AGEs has a catastrophic outcome in diabetes, affecting major tissues of the human body and resulting in various micro- and macrovascular complications associated with diabetes, including retinopathy (Stitt, 2003; Chibber et al., 1997; Tanaka et al., 2000), cataract (Perry et al., 1987), atherosclerosis (Bucala, 1997), nephropathy (Sugiyama et al., 1996; Forbes et al., 2003) and neuropathy (Vlassara et al., 1985). Diabetic embryopathy, another serious complication of diabetes has been attributed to glycation of DNA and histones by reactive sugars, resulting in errors in replication and transcription, thereby promoting mutations (Ahmad, 2005).

Protein glycation end products such as N\textsubscript{6}-(carboxymethyl) lysine (CML), pentosidine and imidazolone accumulate in the kidneys and aortas of diabetic and nondiabetic uraemic patients (Aronson, 2002; Tanji et al., 2000; Wagner et al., 2001; Mao et al., 2003; Kitauchi et al., 2004; Sakata et al., 1999; Niwa et al., 1997a; 1995; 1997b).

Brownlee (2001) carried out research on diabetic complications and proposed four main hypotheses to explain the relationship between hyperglycemia and diabetic complications. These are increased polyol pathway flux; increased AGE formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux. It was the first report to show that overproduction of superoxide by the mitochondrial electron
transport chain was a common outcome of all these mechanisms, resulting in oxidative stress. Further, the concept of ‘hyperglycemic memory’ was coined, which referred to the persistence and progression of hyperglycemia induced microvascular alterations during subsequent periods of normal glucose levels. The increased formation of superoxides during hyperglycemia could induce mutations in mitochondrial DNA. The defective subunits of the electron transport chain encoded by the mutated mitochondrial DNA could lead to increased production of superoxide also at physiological concentrations of glucose, with resulting activation of the four pathways. The study also points towards the need to do gene profiling for the hereditary occurrence of diabetic micro- and macrovascular complications, due to reports of familial clustering in nephropathy and retinopathy (Quinn et al., 1996).

The study by Snieder et al. (2001) showed that HbA1c levels (i.e. a predictor of the risk of microvascular complications) are largely genetically determined and independent of the genes influencing fasting glucose.

The work by Li et al. (2006) was the first to establish the role of CEdG\textsubscript{A,B} (the major known marker for DNA glycation) in the pathogenesis of diabetic nephropathy as well as uraemic vascular complications by immunohistochemistry of kidneys and aorta using an anti-CEdG\textsubscript{A,B} monoclonal antibody. In the kidneys CEdG\textsubscript{A,B} was detected primarily in the nuclei of epithelial, mesangial and endothelial cells of the glomeruli, parietal epithelial as well as tubular cells. More CEdG-positive cells were present in the glomeruli of patients with diabetic nephropathy compared to the controls. In the aortic walls, CEdG\textsubscript{A,B} was mainly localized in the nuclei of macrophages and myofibroblasts, with greater number of CEdG-positive cells detectable in the aorta of hemodialysis (HD) patients and more markedly in diabetic HD patients as compared with control.

Synold et al. (2008) measured the CEdG\textsubscript{A,B} levels in the urine of normal and Streptozotocin induced diabetic Sprague - Dawley rats, employing LC-MS/MS with ESI and using isotopic CEdG\textsubscript{A,B} standard for quantitation. The study showed that 77pg/ml CEdG was excreted by the normal controls, whereas the induction of diabetes increased the urinary
levels ~ 4 fold. They also measured the CEdG_{A,B} levels in human breast
tumour and normal tissue and observed that both stereoisomers were
present at ~ 3-fold higher levels in normal relative to tumor tissue. Within
normal tissue, the levels of CEdG- A and -B were not significantly different,
while in tumor there was a small bias favoring CEdG-A formation.

Kocic et al. (2008) carried out a study on juvenile and adult insulin-
dependent diabetes as well as adults with type 2 diabetes. A fall in plasma
RNase and nuclease activity was observed in juvenile diabetics,
accompanied by increase in circulating nucleic acids and different-sized
oligonucleotides that may contribute towards increase of nucleic acid
“danger motifs” and to the immune stimulation.

Diverse and multiple mtDNA deletions were detected in the skeletal
muscles of ESRD patients. This was attributed to accumulation of uraemic
toxins and impaired free radical scavenging systems, and in turn to
increased oxidative stress. Such stress may result in oxidative damage and
aging-associated mutation of the mitochondrial genome (Lim et al., 2000).

AGEs are also implicated in the pathogenesis of the uraemic
syndrome, for example, dialysis-related amyloidosis, dyslipidemia and
vascular dysfunction (Schwenquer et al., 2001). Genomic damage in
peripheral blood lymphocytes (PBLs), possibly induced by DNA alkylation
was found to be accelerated in uraemic patients (Stooper et al., 1999: 2001).

The 4977 bp deletion of mtDNA in hair follicles was recognized as a
marker for DNA damage in ESRD (Liu et al., 2001). The enhanced genomic
damage observed in mitochondria during ESRD was due to the absence of
protective histones on mtDNA and low efficacy of DNA repair. Since the
DNA repair is impaired in ESRD patients higher incidence of cancer was
observed during HD treatment.

Uraemia is characterized by oxidative (due to iron overload) and
carbonyl stress which promotes production of reactive carbonyl compounds,
that form AGEs and in turn induce DNA damage. Several other theories
were put forward to explain this damage, including that of hypomethylation
(Perna et al., 2006). Kidneys play a key role in excretion of AGEs and
decreased renal function leading to rise in plasma AGEs and their
accumulation in the tissues. Patients subjected to standard HD displayed a 2-fold higher frequency of micronuclei in PBLs than the healthy controls (Sebekova et al., 2007). In contrast, patients on daily HD show lower frequencies of micronuclei, comparable to the controls. Both groups of HD patients display markedly higher plasma AGE levels than the healthy controls, with higher values in the standard HD than daily HD patients. A direct relationship between the frequency of occurrence of micronuclei and the plasma concentrations of CML or imidazolone A (3-DG derived imidazolinone) was evident in PBLs. (Fragedaki et al., 2005). Besides, the genomic damage in PBLs was lower in patients on daily HD than in those on standard HD and this was attributed to lower plasma concentrations of uraemic toxins, including circulating AGEs.

The AGE-RAGE interaction initiates a downstream signaling cascade involving NF-κB, with induction of ROS formation and inflammatory responses to promote development of atherosclerosis. The role of NF-κB was established using the inhibitor dimethylfumarate, which was able to prevent the DNA damage (Schupp et al., 2005). Benfotiamine, a prodrug of thiamine known to reduce AGE levels and microangiopathy was also effective in decreasing the genomic damage in PBLs from patients on HD (Schupp et al., 2008).

Studies on ESRD suggest the following (i) AGEs accumulate and cause enhanced DNA damage, (ii) Precursors of AGEs also induce DNA damage, (iii) a direct correlation exist between plasma AGE levels and DNA damage markers in ESRD, (iv) AGE and RAGE are expressed in tumor cells and the AGE-RAGE interaction participates in tumor invasive and metastatic activity, (v) Higher incidence of cancer is observed in ESRD patients and (vi) ESRD patients with cancer show increased DNA damage and associated DNA mutations (Nagy et al., 2003; Vamvakas et al., 1996).

As stated earlier glycation of proteins may result in denaturation, cellular damage and enzyme inactivation, whereas the nucleotide glycation can cause mutagenesis and apoptosis. AGEs are involved in the physiological complications of various diseases like diabetes and renal failure. Thomalley (2003d) gave the novel concept of the enzymatic defense
against glycation, and related cellular damage, involving enzymes like Glo-1, by oxoaldehydes, reactive carbonyl species and some aldehyde reductase and dehydrogenase isozymes. The enzymatic defense is however not perfect, and glycation adducts of proteins, nucleotides and lipids are still formed under physiological conditions, albeit at low levels. But under certain diseased conditions, particularly diabetes, uraemia, this defense is overcome and AGEs levels rise mainly due to overproduction of MG. Therefore, Glo-I appears to be a major factor associated with risk of developing the vascular complications of diabetes and uraemia (Thornalley, 2003e).

The glyoxalase system is known to be the major detoxifying pathway and comprises of two enzymes, Glo-I and glyoxalase II (Glo-II), that convert MG to D-lactate, through the intermediate S-D-lactoylglutathione (Mearini et al., 2002) (Fig. 5.1). The Glo-I is present in all human tissues and protects them against glycation damage. The specific activity in fetal tissues is however nearly three times higher as compared to adult tissues. Human Glo-I is a dimer and GSH dependent, with its activity proportional to the cellular GSH concentration (Thornalley, 2003e).

Several studies suggest Glo-I expression is enhanced in malignant cells and tissues, most likely to effectively detoxify MG, the level of which rises due to high glycolytic activity (Ranganathan et al., 1995; Thornalley, 1995; Davidson et al., 1999). Increased expression and activity of Glo-I has been reported in various human cancers including those of breast (Rulli et al., 2001), colon (Ranganathan et al., 1993), lung (Illo et al., 1987), bladder and kidneys (Illo et al., 1995), prostate (Samadi et al., 2001) and invasive ovarian carcinomas (Jones et al., 2002) (Fig. 5.2).

MDR limits the efficacy of chemotherapies in various tumours. The resistance of HL-60 cells to anti-tumour agent induced apoptosis was studied by Sakamoto et al. (2000). The cDNA subtractive hybridization with mRNA from tumor cell line U937 and its drug resistant variant UK711 showed that the latter showed overexpression of Glo-I and resistance to anti tumour drugs, etoposide- and adriamycin- caspase activation and apoptosis. Use of a Glo-I inhibitor enhanced the etoposide induced apoptosis in the resistant cells but not in the parental U937 cells. Further, studies on various
Fig. 5.1- The schematic representation of the Glyoxalase enzymatic system.

Fig. 5.2- Glo-1 enzyme activity in human solid tumour cell lines and normal tissue samples (Tsuruo, 2003).
CH₃COCHO
Methylglyoxal

Glyoxalase I

GSH

CH₃CH(OH)CO-SG
S-D-Lactoylglutathione

Glyoxalase II

CH₃CH(OH)CO₂⁻ + H⁺
D-Lactate

H₂O

Normal tissues

Lung
Colon

Cancer cell lines

Lung
Colon
Stomach
Breast
Ovary
Brain
Renal
Prostate

GLO1 Activity (µmol/min/mg protein)
MDR cell lines showed overexpression of Glo-I mRNA and increased Glo-I activity, suggesting the role of overexpression in MDR. Thus, co-treatment of the cell types with anticancer drug as well as the Glo-I inhibitor, BBGC resulted in reversal of MDR and increased sensitivity of the resistant cell type to the drug. It was seen that human lung carcinomas, which showed maximum expression of Glo-I also exhibited highest sensitivity to BBGC and underwent apoptosis when treated with BBGC. The cells with lower Glo-I activity were far less sensitive to the drug (Sakamoto et al., 2001).

The difference in resistance observed in various cell types is in agreement with a study by Castro et al. (1990) on various human tumour cell lines that suggested that every cell line showed different levels of expression of the glutathione-linked enzymes like Glo-I. A unique pattern of the glutathione-linked enzymes was observed suggesting that the resistance phenotypes of the cells differed accordingly.

Glycation by glyoxal, MG and effect of Glo-1 are now believed to play a critical role in aging (Morcos et al., 2005), vascular complications associated with diabetes (Ahmad et al., 2005; Mclellan et al., 1994), renal failure (Agalou et al., 2005; Rabbani et al., 2007), Alzheimer's disease (Chen et al., 2004; Ahmad et al., 2004), tumorigenesis and MDR in cancer chemotherapy (Sakamoto et al., 2001).

The cell lines used in the present study were NCI-H522, NCI-H460, A549, and MG63. These were selected on the basis of their high Glo-I expression with link to MDR characteristics (Sakamoto et al., 2001). The cell lines belonging to the NCI series represent a unique collection of permanent human tumor cell lines established by single laboratory over a period of nearly 16 years. More than 300 cell lines were established and mainly from human lung cancers which were grouped into small cell and non-small cell types (Adi et al., 1996).

NCI-H522 cell type was obtained from a 60 years old Caucasian male. The tumour was a squamous adenocarcinoma with a doubling time of 38.2 h at an inoculation density of 2,00,00. As per information on the homepage of Cancer Genome Project (http://www.sanger.ac.uk/cgi-
bin/genetics/CGP/CGHviewer.cgi), it had a homozygous mutation in the TP53 gene. The other cell line from NCI series, NCI-H460 cell type was also obtained from adenocarcinoma cells from a male (age unknown) (Takahashi et al., 1989). It was a non-small cell lung carcinoma cell line with neuroendocrine properties and a doubling time of 17.8 h and the optimum inoculation density for these cells was 7,500. This cell type was found to have homozygous mutations in the following genes- CDKN2A, KRAS and STK11 as well as a heterozygous mutation in the PIK3CA gene. This cell type was part of the NCI-60 cell line set. A mutation analysis study carried out by Ikediobi et al. (2006) determined the complete sequence of 24 known cancer genes of this cell line. One hundred thirty-seven oncogenic mutations were identified in 14 (APC, BRAF, CDKN2, CTNNB1, HRAS, KRAS, NRAS, SMAD4, PIK3CA, PTEN, RB1, STK11, TP53, and VHL) of the 24 genes. This study was helpful in deciding anti-tumor agents against this cell type (Ikediobi et al., 2006).

As per information available on the homepage of Cancer Genome project, MG63 used in the study was an osteosarcoma cell line obtained from a 14 years old male patient, containing the homozygous mutation in the CDKN2A gene. Zhang et al. (2006) induced MDR in this human osteosarcoma MG63 cell line by exposing the cells to high and gradually increasing doses of cisplatin. Compared to the parental tumor cells, the MG63 resistant cells showed increased expression of MDR markers, P-pg and bcl-2, with p53 expression confirming the occurrence of MDR. In another study, Oda et al. (2000) isolated MDR clones of MG-63 by stepwise selection on exposure to increasing doses of doxorubicin. The final clones selected showed 121-fold higher resistance to the drug than their parental cell lines.

The fourth tumor cell line used for the study was A549, an adenocarcinoma cell line. It was initiated in 1972 by D.J. Giard through explant culture of lung carcinomatous tissue from a 58 years old Caucasian male (Giard et al., 1973). This cell type forms confluent monolayers with Type II pulmonary alveolar morphology and had a doubling time of 22.9 h (at the inoculation density of 7,500). Further
studies by Lieber et al. (1976) revealed that A549 cells could synthesize lecithin with a high percentage of desaturated fatty acids utilizing the cytidine diphosphocholine pathway. The cells were also positive for keratin by immunoperoxidase staining. The cell line showed homozygous mutations in the genes- CDKN2A, KRAS and STK11. Cationized ferritin, a nonspecific absorptive marker, was found to be taken up by the cells in a concentration-, time-, and temperature-dependent fashion. Transferrin, a representative receptor-mediated endocytic marker, was found to be taken up by the cells in a concentration-dependent and competitive fashion. A549 monolayers were polarized, with a greater amount of intracellular transferrin being transported out of the basolateral side of the cells (Foster et al., 1998). Pan et al. (2009) developed a multidrug resistant A549 cell line by exposing the cells to intermittent high doses of cisplatin.

The HL60 cell line used in this study originated from a 35 year old female patient whose leukaemia was first diagnosed as acute progranulocytic (FAB class M3) but on further investigation the diagnosis was altered to acute myeloblastic leukaemia with maturation (FAB class M2) (Dalton et al., 1988). The HL60 cell line continuously proliferates in vitro with a doubling time of 20-48 h (Birnie et al., 1988; Collins et al., 1987).

The overexpression of Glo-I in MDR tumors may have a link to the increased generation of MG in response to DNA repair. Because anticancer drugs provoke the activation of DNA repair as a result poly-(ADP-ribose) polymerase activation, the cells are depleted of NAD⁺. The resulting diminished glyceraldehydes-3-phosphate dehydrogenase activity causes the rise in levels of glyceraldehydes-3-phosphate and other triosephosphates. Triose phosphates are the major precursors for MG. The levels of MG therefore rise in tumor cells, and induce the glycation of DNA and proteins involved with apoptosis. This could be the cause of the cytotoxicity of MG. However, this toxicity of MG maybe overcome by overexpression of Glo-I that restricts MG levels and ensures that the tumour cells become resistant to the drug (Thornalley, 2008).
Pharmacological intervention to inhibit Glo-I was expected to lead to an increase in the cellular concentration of MG in cell systems, and the development of MG toxicity-particularly in proliferating cells and organisms (Vince et al., 1969). Several inhibitors of Glo-I have been designed from analogues of the hemithioacetal substrate based on glutathione derivatives (Thornalley, 1991).

This part of the thesis describes measurements of DNA-AGEs levels in plasma and urine from control and type-2 diabetics, as well as in plasma samples from ESRD patients, carried out using isotopic dilution technique with stable isotopic standards, and by LC-MS/MS tandem mass spectrometry. The Glo-I inhibitor, BBGC gave promising results against MDR (Sakamoto et al., 2001), therefore the present work is an attempt to monitor its effect on the levels of DNA-AGEs in various tumour cell lines overexpressing Glo-I. Another objective of the present study was to examine if inhibiting Glo-I could promote dicarbonyl induced glycation of DNA in tumour cells and test the hypothesis put forward by earlier researches (Sakamoto et al., 2001; Ramasamy et al., 2006).
5.2 RESULTS

A study to examine the possible rise in DNA damage and consequent increase in DNA-AGEs in diabetics and patients of ESRD was undertaken. Blood plasma and urinary levels of 2'-dG, 8-oxodG, MGdG, CEdG<sub>A,B</sub> and GdG were quantified in groups of healthy controls and patients with established diabetes and ESRD.

The method employed for the measurement was optimized in order to restrict artifactual formation of adducts, especially by oxidation on exposure to high pH and elevated temperatures during pre-analytic processing. Care was also taken to ensure prevention of loss of analyte during working, storage (autosampler at 4°C) or over the 24 h period of urine collection at room temperature.

The concentration of the various adducts in plasma and urine samples analyzed using the optimized protocol gave interesting frequency trends. Fig. 5.3 shows the plasma levels of 2'-dG, the important precursor of several DNA-AGEs and 8-oxodG alongwith levels of DNA-AGEs- MGdG, CEdG<sub>A,B</sub>, GdG in blood plasma of healthy controls and type-2 diabetic human subjects. Ninety six percent controls showed plasma 2'-dG median levels at 1.1 nM whereas 30% of the diabetics had the plasma 2'-dG levels of 4.2 nM (Fig. 5.3 (a)). The difference in 2'-dG levels of diabetics as compared to controls was significant. The median levels of 8-oxodG were 0.15 nM for controls and 0.21 nM for diabetics (Fig. 5.3 (b)) but the difference was not statistically significant. MGdG level in type-2 diabetics showed more wide variation and was in the range 0.1-6.9 nM, wherein 48% subjects had plasma levels of the adduct at 1.1 nM and 18% between 2-2.5 nM as compared to 0.8 nM in case of 71% controls (Fig. 5.3 (c)). CEdG<sub>A,B</sub> levels in plasma from control and diabetic subjects were quite comparable and more than 90% population of normal and diabetics had CEdG<sub>A,B</sub> concentration at 0.2 nM (Fig. 5.3 (d)). Fig. 5.3 (e) shows that plasma GdG levels in the controls and diabetics were 0.07 nM and 0.31 nM respectively. The difference in GdG concentration between control and diabetes was found to be highly statistically significant (p<0.001).
Fig. 5.3- The frequency distribution plots for the various adducts of nucleoside oxidation and glycation measured in plasma from healthy controls and type-2 diabetics. The plasma samples from control and type-2 diabetic patients were analyzed by LC-MS/MS and the concentration of (a) deoxyguanosine, oxidation adduct (b) 8-oxodG, and glycation adducts (c) MGdG, (d) CEdG$_{A,B}$, (e) GdG was estimated and plotted against frequency of occurrence of those concentrations in the control and diabetic subjects.
### Plasma 2'-dG (nM)

<table>
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<th>n</th>
<th>Median</th>
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<td>28</td>
<td>1.1</td>
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<tr>
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<td>56</td>
<td>4.2</td>
<td>0.8-13.7</td>
<td>&lt;0.001  (Mann-Whitney)</td>
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### Plasma 8-oxodG (nM)

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<td>0.15</td>
<td>0.04-0.39</td>
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<tr>
<td>Diabetic</td>
<td>52</td>
<td>0.21</td>
<td>0.01-0.46</td>
<td>0.08 (t-test)</td>
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### Plasma GdG (nM)

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<th>Median</th>
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<tbody>
<tr>
<td>Control</td>
<td>28</td>
<td>0.07</td>
<td>0.04-0.20</td>
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<tr>
<td>Diabetic</td>
<td>56</td>
<td>0.31</td>
<td>0.14-0.61</td>
<td>&lt;0.001  (Mann-Whitney)</td>
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### Plasma M6dG (nM)

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<td>0.18</td>
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<td>Diabetic</td>
<td>48</td>
<td>0.20</td>
<td>0.02-14.4</td>
<td>0.104 (Mann-Whitney)</td>
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</tbody>
</table>

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(a) [Plasma 2'-dG](nM)

(b) [Plasma 8-oxodG](nM)

(c) [Plasma GdG](nM)

(d) [Plasma M6dG](nM)

(e) [Plasma CEdG](nM)
Urine samples were also analyzed for the DNA-AGEs. The results in Fig. 5.4 (a) show that 70% of control but only 58% of diabetics had 2'-dG levels below 2 nM. Fig. 5.4 (b) shows 35% diabetics had median 8-oxodG at 9.6 nM while the controls showed levels of 11.6 nM and the difference between control and diabetic levels was statistically significant (p < 0.05). MGdG levels in urine from type-2 diabetics were 3.0 nM in 57% of the patients whereas 78% controls had MGdG levels at 2.7 nM (Fig. 5.4 (c)). Majority of diabetics had CEdGA,B levels at 0.4-0.8 nM while the levels in controls were 0.54 nM (Fig. 5.4 (d)). The difference was not statistically significant. Ninety-six percent of the controls had median urine GdG concentration of 0.13 nM whereas 23% of diabetic patients showed the GdG concentration at 0.8 nM (Fig. 5.4 (e)). Difference between the urinary levels of GdG in controls and type-2 diabetics (p< 0.001) was highly significant. The median values of the various adducts in plasma and urine of control and diabetics are summarized in Table 5.1. The data shows that among the DNA-AGEs, MGdG occurs in highest concentration but the levels of the adduct were unaffected neither in plasma nor urine in the diabetics. On the other hand diabetics contained high level of GdG in the plasma and excreted over 6-fold higher concentration of the adduct in urine.

Clinically creatinine clearance rate is used to measure glomerular filtration rate (GFR) and in turn the overall kidney function. It was therefore considered of interest to investigate whether a relationship exist between creatinine clearance rate in patients of type-2 diabetes and the clearance of the DNA-AGEs from circulation. The creatinine clearance rate of healthy controls was 94.1±32.6 ml/min/1.73m² which was markedly higher as compared to 86.1±23.7 ml/min/1.73m² observed in case of diabetics without nephropathy and 49.7±31.4 ml/min/1.73m² for diabetics with nephropathy. The plots of creatinine clearance rate against plasma MGdG, CEdG_A,B and GdG concentration in control and type-2 diabetics show that the decrease in creatinine clearance rate in patients of type-2 diabetics (especially those with diabetic nephropathy) was accompanied by enhanced levels of the adducts in plasma (Fig. 5.5 (c)). This was estimated by comparing the median values of the various adducts in plasma of control and diabetics. However, the rise
Fig. 5.4 - The frequency distribution plots for the various adducts of nucleoside oxidation and glycation measured in urine from healthy controls and type-2 diabetics. The urine samples from control and type-2 diabetic patients were analyzed by LC-MS/MS and the concentration of (a) deoxyguanosine, oxidation adduct (b) 8-oxodG, and glycation adducts (c) MGdG, (d) CEdG\textsubscript{A,B}, (e) GdG was estimated and plotted against frequency of occurrence of those concentrations in the control and diabetic subjects.
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<td>25</td>
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<td>0.180 (Mann-Whitney)</td>
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**[Urine 2'-dG] (nM)**

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<td>11.6</td>
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<td>9.6</td>
<td>2.9-25.7</td>
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**[Urine 8-oxodG] (nM)**

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<th>Range</th>
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<tbody>
<tr>
<td>Control</td>
<td>26</td>
<td>0.13</td>
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<td>Diabetic</td>
<td>48</td>
<td>0.83</td>
<td>0.12-2.6</td>
<td>&lt;0.001 (Mann-Whitney)</td>
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**[Urine GdG] (nM)**

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<th>Range</th>
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<td>Control</td>
<td>27</td>
<td>2.7</td>
<td>1.2-6.9</td>
<td>—</td>
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<tr>
<td>Diabetic</td>
<td>54</td>
<td>3.0</td>
<td>0.9-6.0</td>
<td>0.888 (Mann-Whitney)</td>
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**[Urine MGdG] (nM)**

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<td>0.12-1.3</td>
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<tr>
<td>Diabetic</td>
<td>54</td>
<td>0.55</td>
<td>0.14-2.4</td>
<td>0.469 (Mann-Whitney)</td>
</tr>
</tbody>
</table>
Table 5.1- The median levels of the DNA glycation and oxidation adducts in plasma and urine obtained from control and type-2 diabetics.

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Plasma concentration (nM)</th>
<th>Urine Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>2'-dG</td>
<td>1.1</td>
<td>4.2</td>
</tr>
<tr>
<td>8-oxodG</td>
<td>0.15</td>
<td>0.21</td>
</tr>
<tr>
<td>MGdG</td>
<td>0.81</td>
<td>1.1</td>
</tr>
<tr>
<td>CEdG_{A,B}</td>
<td>0.18</td>
<td>0.2</td>
</tr>
<tr>
<td>GdG</td>
<td>0.07</td>
<td>0.31</td>
</tr>
</tbody>
</table>

The various significance levels were p-value <0.05, p-value <0.01, p-value <0.001.
in GdG levels in the plasma from diabetics with nephropathy was statistically significant (p<0.001, independent t-test).

Similarly, the median values of excretory MGdG, CEdG<sub>A,B</sub> and GdG concentrations in urine showed that the levels of these adducts were more marked in urine from type-2 diabetes than controls. Further those with diabetic nephropathy showed increase in the urinary levels of the nucleotide AGEs- CEdG<sub>A,B</sub> and GdG (Fig. 5.6 (a), (b) and (c)). The rise in urinary levels of CEdG<sub>A,B</sub> and GdG in case of type-2 diabetics with nephropathy was statistically significant to the limits of p<0.05 and p<0.001 respectively (independent t-test).

In another study, various adducts of oxidative and glycation damage were measured in plasma from normal healthy controls and ESRD subjects. It was seen in Fig. 5.7 (a-c) that in the patients of ESRD, the plasma concentration of 8-oxodG, MGdG and CEdG<sub>A,B</sub> were high as compared to the levels of these adducts in normal healthy controls. However, while GdG concentration also appeared high in plasma of patients with ESRD, the values were not significant when compared to controls (Fig. 5.7 (d)). As evident from Table 5.2, MGdG, 8-oxodG, CEdG<sub>A,B</sub> levels increased 3, 2.8, 2.2 fold, respectively during ESRD.

Studies on various tumor cell lines with Glo-I overexpression and showing MDR characteristics, were carried out in collaboration with Dr. Thomas Santarius, Cancer Genome Project, Welcome Trust Sanger Institute, University of Cambridge, Cambridge, U.K. Dr. Santarius screened the different cell lines for Glo-I activity and expression, and studied them for Glo-I gene amplification by determining relative Glo-I mRNA levels using real time PCR. A gene was called amplified if the relative quantity of that gene was greater than two.

The results obtained showed that NCI-H522 lung cancer cell line showed highest mRNA levels which correlated with maximum Glo-I activity as reported by Sakamoto et al. (2001). This was followed by Osteosarcoma cell line MG63. The lung cancer cell lines, A549 and NCI-H460 showed lower Glo-I mRNA levels (Table 5.3). Further, the IC<sub>50</sub> concentration for the drug, BBGC was determined for the various cell lines as detailed in the
**p<0.01

(a) Plasma eG (nM)

(b) Plasma MG (nM)

(c) Plasma cG (nM)

(d) Plasma Gg (nM)

*p<0.05

Control ESRD
Table 5.2 - Statistical analysis of the plasma nucleotide adducts measured in human subjects from normal healthy controls and ESRD population.

<table>
<thead>
<tr>
<th></th>
<th>Control (Mean ± S.D.)</th>
<th>Range</th>
<th>Median</th>
<th>End Stage Renal Disease (Mean ± S.D.)</th>
<th>Range</th>
<th>Median</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-oxodG</td>
<td>0.255 ± 0.100</td>
<td>0.186-0.370</td>
<td>0.209</td>
<td>0.734 ± 0.323</td>
<td>0.382-1.277</td>
<td>0.707</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MGdG</td>
<td>0.566 ± 0.179</td>
<td>0.440-0.880</td>
<td>0.510</td>
<td>1.734 ± 0.800</td>
<td>0.620-2.680</td>
<td>1.725</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CEdG&lt;sub&gt;A,B&lt;/sub&gt;</td>
<td>0.179 ± 0.079</td>
<td>0.109-0.309</td>
<td>0.172</td>
<td>0.404 ± 0.256</td>
<td>0.173-0.906</td>
<td>0.317</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GdG</td>
<td>0.118 ± 0.023</td>
<td>0.092-0.143</td>
<td>0.114</td>
<td>0.124 ± 0.078</td>
<td>0.057-0.289</td>
<td>0.092</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

The various significance levels were p-value <0.05, p-value <0.01, p-value <0.001.

Table 5.3 - mRNA levels and enzyme activity of Glo-I in various MDR tumor cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; µM</th>
<th>GLO1 mRNA&lt;sup&gt;a&lt;/sup&gt; GLO1/β-actin mRNA</th>
<th>GLO1 activity&lt;sup&gt;b&lt;/sup&gt; µmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG63</td>
<td>3.8</td>
<td>9.5</td>
<td>N.A.</td>
</tr>
<tr>
<td>NCI-H522</td>
<td>7.0</td>
<td>30</td>
<td>8.47±0.10</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>19.8</td>
<td>0.9</td>
<td>0.81±0.02</td>
</tr>
<tr>
<td>A549</td>
<td>23.5</td>
<td>5.9</td>
<td>0.99±0.04</td>
</tr>
</tbody>
</table>

Note: <sup>a</sup> GLO1 mRNA – relative GLO1 mRNA levels measured by real time PCR (determined by Dr. Santarius) and <sup>b</sup> GLO1 activity - as reported by Sakamoto et al. (2001). The values are mean ± S.D. from three independent experiments. N.A.- not available.
material and methods, and it was found to vary between 3.8 μM for MG63 to 23.5 μM for A549 tumour cells (Table 5.3). IC₅₀ represents the concentration of a drug that is required for 50% inhibition of cell accumulation in vitro. Thus, it was expected that cell types with low IC₅₀ will be more sensitive to the action of the inhibitor and as a result have higher cellular MG levels, and consequently increased apoptotic index after inhibitor treatment while cells with high values of IC₅₀ concentration would show decreased sensitivity to the inhibitor.

The real time quantitative PCR studies carried out by Dr. Santarius showed that amplification of the Glo-I gene in the cell lines MG63 and NCI-H522 was two fold or more whereas the cell types A549 and NCI-H460 did not show such enhancement in gene amplification. The study also concluded that there was substantial variation in sensitivity of the cell lines to this drug. The cell lines with the amplification were more sensitive to Glo-I inhibitor treatment as compared to those without amplification (Sakamoto et al., 2001). This is indicated in the low values of IC₅₀ for the drug BBGC in the cell types- MG63 and H522 (those showing amplification of Glo-I) in comparison to those observed in cell types without amplification (A549 and NCI-H460 cell types) (Table 5.3).

A large scale cell culture was carried out for the cells types, in presence or absence of 20 μM Glo-I inhibitor, BBGC for 24 h. After enzymatic hydrolysis of the DNA extracted from the cultures, the hydrolysate was studied using the LC-MS/MS for the various markers of DNA oxidation and glycation.

Fig. 5.8 shows that cell types with amplification have higher levels of the adducts, MGdG, GdG, 8-oxodG. The levels of CEdGₐ,ₜ were however somewhat higher in case of A549 and H460 cells exhibiting no amplification of Glo-I as compared to the MG63. CEdGₐ,ₜ levels in H522 cells were however the highest among the cells studied.

Cell types without amplification of the Glo-I gene showed little change in the levels of MGdG, GdG and 8-oxodG (Fig. 5.8 (a,c,d)), on treatment with the Glo-I inhibitor, except the A549 cell type that showed significant rise in CEdGₐ,ₜ (Fig. 5.8 (b)). The cells with amplification of the Glo-I gene showed
only a modest increase in the formation of the adducts, MGdG and CEdG_{A,B} after inhibitor treatment, although the difference as compared to the controls was however not statistically significant. Increase in the GdG levels in MG63 and H522 cells was observed but the rise was statistically significant only in case of the latter. H522 cells also exhibited a marked increase in the concentration of 8-oxodG in response to BBGC treatment but inspite of the difference of the mean value from the control, the rise was not statistically significant due to large variation between the samples.
5.3 DISCUSSION

Glycation is a recognised contributor to diabetic nephropathy, ESRD and researches have focused on the pathogenic properties of various protein AGEs such as Nε-(carboxymethyl) lysine (CML), pentosidine and imidazolone. Diabetic patients with renal impairment show decreased urinary excretion of fluorescent AGEs and CML accompanied by their elevated serum levels (Tanji et al., 2000; Wagner et al., 2001).

Dicarbonyl compounds like MG and glyoxal are implicated in carbonyl stress, increased formation of AGEs and the development of diabetic nephropathy (Jadidi et al., 2003). Type-2 diabetes is known to be associated with increased MG and glyoxal levels (McLellan et al., 1994) as well as oxidative stress (Ceriello et al., 2004). Hyperglycemia in diabetes results in excessive accumulation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate which are precursors of MG. Jadidi et al. (2003) reported that MG derived protein AGES - methylglyoxal derived hydroimidazolone Nε-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) and Nε-(1-carboxyethyl) lysine (CEL) increased two-fold in glomerular protein of Streptozotocin (STZ) induced diabetic rats. Little work is however available on DNA-AGEs as a possible measure of diabetes associated complications and this study was therefore undertaken.

Jakus and Rietbrock (2004) observed that Hb-AGEs represent only the metabolic status, whereas elevated serum AGES correlate better with the complications associated with diabetes and it was proposed that the levels of serum AGES may prove to be more useful as early biochemical markers of microangiopathy in diabetics. The increased levels of protein AGES in the serum have been shown to reflect the progression of morphological changes in the basal membrane thickness and mesangial fraction in the kidney (Berg et al., 1997).

Among the DNA-AGEs, CEdG_{A,B} was the first to be identified as a major product of glycation damage to nucleotides in in vitro experiments (Ochs and Severin, 1994). A competitive ELISA using a monoclonal anti-CEdG antibody was used to measure CEdG_{A,B} levels in normal human urine (Schneider et al., 2004). The work suggested that glycation induced DNA
damage leading to the formation of the AGE indeed occurs \textit{in vivo}. In a more recent study, Li et al. (2006) reported the accumulation of CEdG\textsubscript{A,B} in the kidneys of diabetics and aorta of HD patients. Synold et al. (2008), in a recent study measured the CEdG\textsubscript{A,B} levels in the urine of normal and Streptozoticin induced diabetic Sprague - Dawley rats, employing LC-MS/MS with ESI and using isotopic CEdG\textsubscript{A,B} standard for quantitation. The study showed that 77pg/ml CEdG was excreted by control rats whereas the induction of diabetes increased the urinary levels ~ 4 fold.

The ability of the kidney to filter the toxic AGEs out of the body was affected in case of diabetic nephropathy where the glomerular filtration rate (as represented by the creatinine clearance rate) was decreased. As a result the levels of serum AGEs in diabetics with nephropathy were higher as compared to those of patients without the complication (Ono et al., 1998).

Protein AGEs are also known to accumulate in plasma during ESRD and promote damage \textit{in vivo} (Dawnay, 2003; Odani et al., 1999). Besides, a direct correlation was observed between plasma protein AGE levels (CML and imidazolone) and DNA damage in ESRD as estimated by the micronuclei frequency assay (Fragedaki et al., 2005). It was suggested that higher incidence of cancer observed in ESRD patients may result from increased AGE induced DNA damage and resulting mutations (Sebekova et al., 2007). Plasma AGEs derived from proteins are removed by the kidney, and decreased renal function leads to rise in plasma AGEs and their accumulation in various tissues (Monnier et al., 1992; Schinzel et al., 2001).

A study was therefore carried out to measure the levels of DNA-AGEs in biological fluids from healthy controls and patients of type-2 diabetes and ESRD. All possible efforts were made to ensure specificity/accuracy in the analyses. The ultrafiltrates of the biological fluids were spiked with isotopic standards, ensuring accurate peak identification. Stable isotopic dilution analysis liquid chromatography with tandem mass spectrometric detection (SILC-MS/MS) employed for the analysis of the plasma and urine samples did not necessitate tedious sample processing. Artifactual oxidation was minimized and analysis time reduced since ultrafiltration was the only purification step employed in the sample processing. Switching of valves
where the flow was directed towards waste for the initial 4 min of the run, prevented impurities from entering the mass spectrometer. This was an improvement over the earlier procedures used for preparation and analysis of biological samples, wherein solid phase extraction was used for sample clean up (Germadnik et al., 1997; Ravanat et al., 1998) prior to LC-MS/MS quantitation. Under the conditions used the limit of detection for the adducts MGdG, CEdGAB, GdG and 8-oxodG were 0.08 pmol, 0.02 pmol, 0.03 pmol and 0.06 pmol respectively (Table 2.1).

The study is a first of its kind in which the major markers of oxidative and glycation damage to DNA have been measured in human subjects using stable isotopic dilution analysis liquid chromatography with tandem mass spectrometric detection.

Data in Fig. 5.3 and 5.4 (b-d) shows a remarkable increase especially of GdG both in plasma and urine of diabetics. Diabetes is associated with accumulation of MG and glyoxal (McLellan et al., 1994, Jadidi et al., 2003). Increased levels of 2'-dG along with dicarbonyls like MG and glyoxal could promote the accumulation of nucleotide glycation adducts like MGdG, CEdGAB and GdG in plasma and excretion in urine from diabetics. Jadidi et al. (2003) showed that plasma levels of glyoxal in diabetic rats were moderately higher as compared to MG and 3-deoxyglucosone.

It is known that hyperglycemia is the initiating event for both intracellular and extracellular AGE formation in diabetics (Brownlee, 2001). Thornalley et al. (1999) suggested multiple routes of glyoxal formation from glucose, which explain the enhanced formation of glyoxal in diabetes (as a result maximum generation of GdG). Besides, the intracellular autooxidation of glucose may also promote glyoxal formation that could further contribute towards enhanced AGE formation (Brownlee, 2001). Manini et al. (2006) demonstrated that removal of oxygen decreased glyoxal formation. Besides, Murata-Kamiya et al. (1997) stated that glyoxal generated from DNA and DNA precursors, immediately reacts with the DNA and the precursors to cause base modifications resulting in even higher GdG levels.

Fig 5.3 shows that 2'-dG levels in the plasma of type-2 diabetics were enhanced ~ 4 fold. This rise in nucleotide levels could be attributed to DNA
damage and/or its decreased renal clearance in patients with type-2 diabetes. The enhanced levels of 2'-dG may also not relate to increased degradation of circulating nucleic acids/polynucleotides since nucleases seem to be inhibited in diabetes. Kocic et al. (2008) observed that activity of RNase and a specific nuclease in the plasma from diabetics was significantly decreased as a result of hyperglycemia.

The reported concentrations of MG and glyoxal in human blood plasma from normal individuals were in the range of 100-120 nM (Beisswenger et al., 1999; Strzinek et al., 1972) while cellular concentrations of MG ranged between 1-5 μM and that of glyoxal between 0.1-1 μM (Dobler et al., 2006). Under conditions of limiting α-oxoaldehyde, prevalent in vivo, the major nucleotide AGEs are the imidazopurinone derivatives, MGdG (Schneider et al., 1998; Papoulis et al., 1995) and GdG (Kasai et al., 1998). The adducts are unstable at high pH and temperatures and necessitate mild conditions of DNA extraction and hydrolysis (Thornalley, 2008). The earlier researchers (Seidel and Pischetsrieder, 1998a; Frischmann et al., 2005) carried out the incubation reaction of DNA/deoxyguanosine with L-ascorbic acid, L-dehydroascorbic acid, D-glucose, D/L-glyceraldehyde, MG or dihydroxyacetone at temperatures (i.e. 40 and 70°C) that could promote the degradation of the labile cyclic adduct (MGdG). Therefore, the observation that CEdGA,B was the major adduct formed on glycation of DNA in vitro and in vivo therefore needs to be reexamined.

The order of nucleotide adduct levels in plasma from type-2 diabetics was: MGdG>GdG>8-oxodG>CEdGA,B, whereas in urine samples from type-2 diabetics it was 8-oxodG>MGdG>GdG>CEdGA,B. Artifactual 8-oxodG formation occurring during the 24 h time period of urine collection may contribute towards the high levels of the adduct which may result in increased 8-oxodG concentration in the urine.

Our CEdGA,B estimates in plasma from normal healthy controls were in the range - 1.37-185.39 ng / mg creatinine, whereas those in urine were in the range - 0.058-0.540 ng / mg creatinine (Fig. 5.3 and 5.4). An earlier study showed that CEdGA,B levels in the urine of normal healthy human subjects determined using immunoassay were higher and in the range - 1.2-
117 ng equiv./ mg creatinine (Schneider et al., 2004). The high values of the
adduct reported in the study may have resulted from the poor specificity of
the monoclonal antibody used. Besides MGdG maybe transformed to
CEdGA,B in the samples during the pre-analysis processing due to exposure
to high pH.

A procedure for the successful isolation of both MGdG as well as
CEdGA,B for use as standards during quantitative studies was developed.
There have been few quantitative estimates of nucleotide AGEs in
mammalian cells. Thoralley (2008) showed the presence of MGdG, GdG as
well as 8-oxodG in DNA extracted from human MNLs, with maximum levels
being those of MGdG. Similar results were obtained in the present study on
DNA obtained from MNLs (Fig. 4.9).

Figures 5.3 and 5.4 show that MGdG was the major AGE both in
plasma and urine. There was however no significant increase in MGdG
levels either in plasma or urine of diabetic patients. Similarly, no increase
was observed in plasma and urine CEdGA,B in diabetics. As has been
mentioned, rats rendered diabetic with STZ excrete four-fold higher CEdG
compared to controls (Synold et al., 2008). Apparently diabetics do not
exhibit enhanced excretion of adducts as observed in case of diabetic rats.

The concentration of all the adducts (Table 5.1) excreted in urine
were higher as compared to those in plasma, presumably due to
concentration of blood plasma by the kidney. On comparing the median
values for the adducts, it is evident that in case of type-2 diabetics the
urinary levels of only GdG increased a significant 6.3-fold while those of 2'-
dG, MGdG, 8-oxodG and CEdGA,B showed no such increase in diabetics as
compared to controls. On the other hand, the plasma concentration of 2'-dG
and GdG increased 3.8 and 4.4 fold in case of diabetics. The levels of
CEdGA,B and MGdG registered only a marginal rise.

Our data in Fig. 5.3 and 5.4 shows that although in samples obtained
from type-2 diabetics, the levels of the MGdG adduct are higher as
compared to the other DNA glycation adducts (CEdGA,B and GdG) analysed,
but these did not differ significantly from the levels observed in normal
healthy controls. This maybe related to a high variation among the
individuals and lack of very large sample size. GdG levels in the type-2 diabetics were significantly elevated in both plasma and urine as compared to the controls suggesting remarkable enhancement in the formation of GdG. Our data on MDR tumour cells also supports this observation. The H522 cell type having highest Glo-I activity also showed significant rise in GdG levels after the inhibitor treatment (Fig. 5.8). Therefore, this study further suggests that GdG maybe considered as a major biomarker for studying diabetes.

Accumulation of AGEs in proteins has a catastrophic outcome in diabetes, affecting major tissues of the human body and resulting in various micro- and macrovascular complications associated with diabetes including nephropathy (Sugiyama et al., 1996; Forbes et al., 2003). The results in Figures 5.5 and 5.6 show that diabetic nephropathy is accompanied by accumulation of DNA-AGEs like GdG in the plasma with significantly enhanced excretory levels of GdG and CEdG_{A,B} in the urine. This correlates with the findings of Li et al. (2006) who demonstrated an increased accumulation of CEdG_{A,B} in the kidneys of diabetic patients with nephropathy and the aortas of hemodialysis patients. The number of CEdG-positive cells in glomeruli was significantly increased in diabetic nephropathy suggesting that CEdG_{A,B}, a DNA-linked AGE, may lead to loss of genetic integrity in the kidney of patients with diabetic nephropathy.

The data in Table 5.2 suggests that there is significant accumulation of the DNA-AGEs – MGdG and CEdG_{A,B} but not GdG in plasma obtained from patients of ESRD. These observations need to be substantiated with additional analysis on ESRD patients as only 13 patients were available for the present study. Several other causes of renal failure in ESRD have been stated including glomerulonephritis, polycystic kidney disease, interstitial nephritis, reflux nephropathy, besides diabetes (Fragedaki et al., 2005). It is noteworthy that the patients recruited for the present study were not diabetic. In these patients the DNA damage and resultant DNA-AGE formation may therefore be influenced by factors other than those that influence DNA damage in diabetes. As a result the order of nucleotide adduct level in plasma from patients of ESRD was different from that observed in type-2 diabetics.
It has been shown that the plasma concentration of AGE precursors—i.e. dicarbonyls like MG and glyoxal are elevated during uraemia in ESRD patients (Miyata et al., 1999). Their accumulation is not due to impaired renal clearance, instead multiple routes channel together to increase formation of the cellular triose phosphates, the precursors for MG formation (Dawnay, 2003; Odani et al., 1999). Fig. 5.7 shows that plasma samples from ESRD patients have greatly enhanced MGdG levels (Mean ±S.D.=1.734 ± 0.8 nM as compared to those of normal healthy controls (Mean ±S.D. = 0.566 ± 0.179 nM), (p=0.004). Similarly the degradation product of MGdG adduct, the carboxyethyl derivative of deoxyguanosine (CEdGA,B) was also significantly elevated in the plasma samples from ESRD (Mean ±S.D. = 0.404 ±0.256). The order of nucleoside adduct levels in plasma from ESRD patients was - MGdG>8-oxodG>CEdGA,B>GdG. Plasma samples obtained from both type-2 diabetics as well as ESRD patients showed high levels of MGdG (Fig. 5.7), although the difference from controls in the former was not statistically significant. It has also been shown in earlier studies that free protein-AGEs are present in blood plasma of patients with a relatively high renal clearance and accumulate markedly in ESRD (Meerwaldt et al., 2009). Therefore, significant accumulation of the DNA-AGEs – MGdG and CEdG_A,B was observed in the plasma from ESRD patients (Fig. 5.7)

Several of the previous researches focused on the 8-oxodG and 8-OHdG measurements in urine from control and diabetics (Weimann et al., 2001; Cooke et al., 2008). Our results of urinary 8-oxodG levels in controls (i.e.15.2 ± 8.8 nM) are lower than the previously reported value (i.e. 28 ±2 nM /24 h) as determined by HPLC-MS/MS with ESI (Weimann et al., 2002). The high values reported by Weimann et al. (2002) could arise, as discussed earlier, due to the additional heating step used by them during the sample preparation in order to dissolve the precipitate formed.

This study provides evidence for in vivo formation of MG and glyoxal derived nucleotide AGEs in human subjects as well as oxidation products and that these adduct levels are greatly elevated during disease states like type-2 Diabetes and ESRD, respectively.
It has been shown that some MDR tumour cells use the Glo-I enzymatic machinery as a defense tool against glycation-mediated apoptosis (Tsuruo et al., 2003). Therefore, the effect of the Glo-I inhibitor (BBGC) on the induction of MG mediated glycation in DNA in the MDR tumour cells was examined. The results obtained show that cell types with amplification of Glo-I gene and thus, enhanced Glo-I activity were found to be more sensitive to the inhibitor treatment as seen in case of NCI-H522 and MG63 tumour cells. This was in coherence with earlier reports on MDR tumour cells (Sakamoto et al., 2001). The results in Fig. 5.8 show that the GdG nucleotide adduct levels in cell lines with Glo-I gene amplification (NCI-H522) and CEdG_{A,B} levels in A549 (cell type without amplification) were significantly elevated after inhibitor treatment.

MG is known to enhance cisplatin induced apoptosis by activating protein kinase Cδ and reports also show that tumor necrosis factor (TNF) induces a substantial increase in intracellular levels of MG and a specific MG-derived AGE (Tsuruo et al., 2003). Besides MG modification was involved in Hsp27 oligomerization to prevent cytochrome c-mediated caspase activation during apoptosis (Sakamoto et al., 2002). This suggests that MG modification of proteins could be a targeted process and that MG may function as a signal molecule during the regulation of cell death. Since inhibition of Glo-I is likely to cause accumulation of MG, and in turn induce DNA modification and protein cross-links, DNA damage or inactivation of some anti-apoptotic proteins by MG could be an initial activator of apoptosis (Tsuruo et al., 2003).

It was anticipated that levels of MGdG and CEdG_{A,B} and the other MG derived AGES would rise after inhibitor treatment. No significant increase in MGdG or CEdG_{A,B} was however observed. It is not unlikely that enhanced generation of MG as a result of BBGC induced inhibition of Glo-I may not reach DNA. Instead the generated MG may rapidly react with proteins and induce apoptosis through pathways not involving DNA-AGES.