MUT/2015/02 – Updated (June 2015)

The Potential Role of Oxidative Damage in Alcohol's Mutagenic and Carcinogenic Mode of Action – An Overview

Introduction

1. As an adjunct to the ongoing review of alcohol consumption and cancer by COC, the COM reviewed a paper (MUT/2014/02) summarising studies since 2000 investigating the genotoxicity of alcohol, with a view to updating the current statement (COM 2000). The paper focussed on the genotoxicity of alcoholic beverages in consumers, mutagenicity tests of ethanol ~~(EtOH)~~ and acetaldehyde (AA), its primary metabolite and on the generation of DNA adducts by EtOH. In the review of the literature, a number of reports which examined the potential for alcohol to cause oxidative damage to DNA and proteins were retrieved. At the meeting in October 2014, COM were presented with brief summaries of these studies. It was decided that a detailed or systematic review of this topic was not necessary. However it was suggested that an overview of the potential role of oxidative damage, and the role of CYP2E1 in particular, would provide useful insight into the overall mode of action of alcohol-induced mutagenesis and carcinogenesis. It is considered that it might be helpful to include a short section on this topic in the COM's statement on alcohol and mutagenicity.

Oxidative damage mode of action in [hepato]carcinogenesis

2. There is an extensive body of research which associates the toxicity of alcohol in alcoholic liver disease ~~(ALD)~~ with the generation of reactive oxygen species (ROS), the resultant lipid peroxidation and free-radical mediated damage (Albano 2006). In animal models, particularly in animals fed diets high in polyunsaturated fatty acids, pathological liver damage correlated with markers of lipid peroxidation such as manganese superoxide dismutase (MnSOD), glutathione peroxidise and catalase levels (Nanji et al 1994; Polavarapu et al 1998). Chronic alcohol consumption is associated with inflammation, which is considered to be a major factor in the development of alcoholic hepatitis (Seitz and Stickel 2006).

3. The relationship between inflammation, oxidative stress, generation of ROS and carcinogenesis is well established for a number of cancers including lung, liver and pancreas (Nair et al 2007; Filaire et al 2013; Valavanadis et al 2013; Seitz and Stickel 2006; Ling et al 2014). Inflammatory reactions can generate ROS and reactive nitrogen species via macrophage activation, which are believed to cause DNA damage directly (via deamination or guanine oxidation), or indirectly via the decomposition of lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) (Lonkar and Dedon 2010). These can result in the production of DNA reactive electrophiles and subsequent generation of hydroxyethyl radicals (HER) and exocyclic DNA etheno adducts (e.g. N^6-etheno-2'-deoxyadenosine, εdA; N^4-etheno-2'deoxycytidine, εdC) (Bartsch and Nair 2004; Blair 2008).

4. Etheno adducts are considered to be highly mutagenic (Barbin 2000). Evidence for this includes, the detection of persistent adducts which arise during

vinyl chloride and urethane carcinogenesis (Barbin et al 2000; Swenberg et al 1992). The mutations identified following alcohol exposure include: AT→GC transitions and AT→TA and AT→CG transversions induced by εdA and CG-AT transversions and CG→TA transitions induced by εdC (summarised in Linhart et al 2014- ANNEX 1). It is also suggested that etheno adducts are preferentially formed in codon 249 of TP53 (Hu et al 2002).

5. There is a large database of studies which have investigated exocyclic DNA adducts, such as those generated by MDA and HNE, as biomarkers of oxidative stress in both rodent and human studies. There are strong associations between the generation of these adducts and human cancers considered to have an inflammatory component in their aetiology (Nair et al 2007). As well as etheno adducts, there are many adducts considered to arise from an oxidative mode of action (MOA) of alcohol or acetaldehyde exposure – these include N^2-ethyl-2'-deoxyguanosine (Singh et al 2012) and 8-oxoguanine (8-hydroxyguanine) (Hirano 2011).

6. 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) adducts are repaired by~~ by~~ 8-~~O~~oxoguanine glycosylase (OGG1) and there are examples of both being used as biomarkers to estimate ROS-induced DNA damage in studies investigating alcohol induced toxicity (Hirano et al 2009; Guo et al 2008). However as repair of 8-OH-dG is efficient, it is considered to be more useful as a biomarker of exposure rather than of effect. ~~may not be a good marker of oxidative damage.~~

7. There is evidence from s~~S~~tudies in both animals and humans to support the hypothesis that free-radical oxidative damage is of importance in alcohol-induced hepatotoxicity generally and that chronic inflammation is likely to contribute to the responses observed (Albano 2006; Bartsch and Nair 2006; Seitz and Stickel 2006).

Alcohol and Cytochrome P450 2E1

8. Cytochrome P450 2E1 (CYP2E1) is widespread amongst mammalian species and is considered to be of particular importance in toxicology as it is known to metabolise a wide variety of xenobiotics, including many which result in the production of toxic metabolites (Novak and Woodcroft 2000). It is transcriptionally and post-transcriptionally regulated, and induced by a wide range of substances including ethanol. CYP2E1 is primarily localised in the liver. Increased expression, ~~up to~~of 10-20-fold, has been demonstrated in rats following treatment with ethanol (Ingelman-Sundberg et al 1993; Albano et al 1996). ~~and i~~It wa~~i~~s elevated by up to 10-fold in humans following high, chronic alcohol consumption (166 ± 10.8 g/day) ~~by up to 10-fold~~in ~~h~~the liver (Dupont et al 1998) and activity increased by approximately 4-fold in healthy volunteers given 40 g/day for 4 weeks (Oneta et al 2002) ~~in the liver~~. It is also induced in some extrahepatic tissues, including the oesophagus and intestines (Seitz and Wang 2013). It is estimated to have a turnover rate of approximately 2.5 days in humans – this makes it possible to monitor changes during alcoholic withdrawal with relative accuracy.

9. CYP2E1 has a high rate of NADPH oxidase activity which results in enhanced production of ROS (O_2^- and H_2O_2) from oxygen~~O_2~~ and consequent generation of HER and lipid peroxidation products (Seitz and Stickel 2007). This is evidenced *in vitro* and *in vivo,* in both animal models and in humans. CYP2E1 metabolises

alcohol to acetaldehyde, with concomitant generation of ROS (Seitz and Wang 2013).

10. A role for CYP2E1 in alcohol-induced liver damage is well established in animal models. For example, key studies in rats showed that the administration of CYP2E1 inhibitors (diallyl sulphide, ~~DAS~~ and phenyliocyanate ~~PIC)~~ reduced lipid peroxidation, liver damage and hydroxyethyl radicals induced by ethanol treatment (Morimoto et al 1995; Albano et al 1996). Oxidative damage, as measured by oxidative DNA adducts, mutagenic apurinic and a~~/~~pyrimidinic sites, and expression of base excision DNA repair genes, was not seen in the livers of CYP2E1 null mice compared to wild-type mice and rats given EtOH orally in liquid diet (Bradford et al 2005). In mice, transgenic for human CYP2E1, liver damage following administration of an alcohol rich diet (30% of calories) is more pronounced than in non-transgenic mice (Morgan et al 2002). In a rat model of alcoholic liver disease (genetically obese Zucker (fa/fa) rats and their lean littermates (Fa +/-), a correlation between adducts (εdA, εdC) and CYP2E1 localisation was demonstrated in the liver following alcohol treatment. However there was no significant difference in hepatic 4-HNE protein adducts in rats (Wang et al 2009).

11. Results of early long-term bioassays suggested that alcohol is not itself a carcinogen when administered under experimental conditions to animals but more recent studies report increased tumours in treated rats and mice (summarised in IARC 2010; Seitz and Stickel 2006). The ability of ethanol ~~EtOH~~ to enhance chemically-induced carcinogenesis such as that of nitrosamines is well documented (IARC 2010) and it is proposed that this co-carcinogenic activity is mediated via induction of oxidative metabolism by CYP2E1 which increases the generation of DNA reactive metabolites and exacerbation of DNA damage (~~IARC 2010;~~ Seitz and Stickel 2006).

12. There are a number of studies which have examined the potential of alcohol to enhance mutagenicity or carcinogenicity of nitrosamines in animal models. For example,

- In a study examining the impact of ~~EtOH~~ ethanol administration on N-nitrosodimethylamine (NDMA) induced effects, rats were given ~~EtOH~~ ethanol as an acute dose (5g/kg) or for 4 weeks in liquid diet (5%w/v) with or without an acute dose of NDMA (4mg/kg). ~~EtOH~~ Ethanol treatment alone increased CYP2E1 protein levels and NDMA demethylase activity. Enhanced formation of εdA and εdC were reported. NDMA alone increased O^6-Me-G levels and this was further increased by administration of ~~EtOH~~ ethanol (Navasumrit et 2001):
- In rats treated with alcohol in drinking water with and without three daily doses of N-nitrosomethylbenz~~e~~ylamine (NMBA 0.5 mg/kg) for 5 weeks, increased levels of CYP enzymes in the liver and oesophagus, including CYP2E1 (Tatematsu et al 2013). It is suggested that localised CYP2E1 induction contributes to nitrosamine activation in the oesophagus [
- Additionally, microsomes from alcohol treated rats significantly enhanced mutagenicity of a range of nitrosmaines (NPYR, DMN, DEN, NMBA) in the Ames test Mori et al 2002]

- In humans, the synergistic carcinogenic effect of alcohol and tobacco may also be explained, to some extent, by the induction of CYP2E1 by alcohol, particularly in extra-hepatic tissues such as the oesophagus. This mechanism was examined by COC as part of their review of the effects of mixtures and co-administrations on cancer in man (CC/08/10~~— ANNEX 1~~). This association was also described by IARC (2010).

135. Linhart et al (2014~~, ANNEX 1~~) provides an overview of CYP2E1, the generation of ROS and etheno-adducts focussing on alcohol as an example. As well as describing the role of inflammatory processes and studies in which exocyclic DNA adducts are generated from lipid peroxidation as by-products from CYP2E1 induction, strong correlations between CYP2E1 induction and etheno-adduct formation in the liver are described. Furthermore, investigations which have assessed CYP2E1 in patients with oesophageal and colorectal tumours also point towards a role for CYP2E1 induction in their aetiology.

Investigative studies in humans

146. There are a number of papers which have investigated the relationship between alcohol consumption in humans, CYP2E1 induction/expression and the generation of etheno adducts. CYP2E1 activity in man is often measured by chlorzoxazone (CHZ) metabolism, a substrate considered to be highly specific for this enzyme.

157. Oneta et al (2002) examined the kinetics of CYP2E1 in non-alcoholic and alcoholic males. Five healthy male volunteers were given 40 g ethanol/day for 4 weeks. CYP2E1 activity was increased, measured by CHZ metabolism after 1 week of ethanol consumption and continued to increase over the 4 week dosing period. In a second arm of the study, CYP2E1 activity was significantly reduced in alcoholics (n=5) 3 days after alcohol withdrawal, although the results were variable.

168. In a study examining CYP2E1 activity in alcoholics, 40/51 patients had elevated CYP2E1 (as measured by CHZ metabolism) – the remaining were within control range (Dupont et al 1998). It was also reported that alcohol increased IgG against protein-adducts of hydroxyethyl free radicals and a correlation with CYP2E1 inducibility is suggested. The authors speculate on a link between induction of CYP2E1 and the development of alcoholic liver disease.

179. In a further study by the same group, CYP2E1 by CHZ metabolism was assessed in 40 chronic alcoholics and 18 controls (< 20g alcohol/day) (Dupont et al 2000). Parameters of oxidative stress, plasma lipid peroxides (LPO) and plasma vitamin E were determined and antibodies to HER and MDA adducts provided a measure of oxidative damage.~~—~~ CYP2E1 activity was significantly correlated with alcohol consumption and plasma levels, though the association was weak. However, the markers of oxidative stress in alcoholics, although discussed by the authors in terms of being increased in alcoholics, (by up to 20%) were not statistically different from controls (with exception of HER adducts).

1820. CYP2E1 and CYP3A activities were assessed in 20 subjects with moderate alcohol consumption (140-210 g/week, equivalent to 20-30 g/day) and compared to

20 age, gender BMI matched, non-drinker controls (Liangpunsakul et al 2005). mRNA of each CYP was measured in peripheral lymphocytes and enzyme activity was measured by clearance of midazolam (MDZ) and CHZ for CYP3A and 2E1 respectively. CYP2E1 mRNA and clearance of CHZ were significantly increased by moderate alcohol consumption, and whilst CYP3A4 mRNA was increased, MDZ clearance and PK parameters were unaffected.

19~~21~~. In a study using liver biopsy samples from patients with ~~ALD~~alcoholic liver disease, it was shown that there was a strong correlation between CYP2E1 expression, protein bound 4-HNE and the formation of etheno-DNA adducts (Wang et al 2009). The association was corroborated with results from a rat model of ~~ALD~~alcoholic liver disease as described above. In HepG2 cells transfected with human CYP2E1, immunohistochemical detection of εdA was noted after EtOH administration but not in vector mock cHepG2 cells. Formation of εdA was prevented by the CYP2E1 inhibitor chlormethiazole (CMZ).

20~~2~~. An evaluation of oxidative damage endpoints was undertaken in patients with alcohol dependency (Chinese population sample:~~ -~~ 79 alcoholics – mean consumption 196.5 g/day, 63 non-drinker controls). Levels of 8-OHdG and MDA were measured in blood taken at baseline and 1 week after alcohol detoxification to investigate changes in these markers during alcohol withdrawal syndrome (Chen et al 2011). At baseline, MDA and 8-OH-dG were higher in alcoholics compared to controls. Following alcohol withdrawal, MDA was reported to be reduced but 8-OH-dG was increased. However the authors state that the 8-OH-dG levels may be confounded by factors such as age, smoking and vitamin status.

21~~3~~. Bianchini et al (2001) examined the relationship between alcohol consumption and 8-OH-dG adducts in lymphocytes from groups of non-smoking women from different parts of Europe (n= 24, 27, 28 and 36). Daily alcohol consumption varied between groups (regions) (1.0±2 – 13.1 ±15.7). There was an inverse relationship between adducts and consumption in all four study groups which persisted after re-adjusting for possible confounders such as fruit and vegetable consumption, plasma carot~~i~~enoids.

22~~4~~. An investigation of human oesophageal tissue biopsies aimed to examine the hypothesis that CYP2E1 induction may also contribute to alcohol's MOA in oesophageal cancers (Millionig et al 2011). The study aimed to determine if chronic alcohol consumption induces CYP2E1 in oesophagus similar to the liver and if there is a correlation between induction, exocyclic etheno adducts and a cell proliferation marker, Ki67 (all were measured using immunohistochemical techniques). Tissues were taken from 37 patients with oesophageal cancer (2 abstainers~~teetotallers~~, 10 people consuming ~~1~~0-60 g/day, 25 people consuming more than~~ >~~ 60 g/day) and compared with tissues from 16 from non-cancer patients (12 ~~teetotallers~~abstainers, 4 people consuming 0-25g/day). Smoking status was also taken into consideration. CYP2E1 expression and etheno-adduct levels were strongly correlated in the individual samples examined. Smokers who drank had a significantly higher proliferation rate, CYP2E1 expression and nuclei containing etheno adducts than those that didn't – similarly patients with cancer had higher levels of all three markers compared to those without cancer. Alcohol consumption correlated with CYP2E1 expression and etheno adducts but not proliferation. It should be noted that there

~~Whilst this study looks robust there~~ are also reports of a number of other CYP forms (e.g. CYP2C8, CYP3A4, CYP3A5), elevated in oesophageal cancer, and therefore the role of CYP2E1 is not clear cut (Bergheim et al 2007).

The role of CYP2E1 Polymorphisms in alcohol's mode of action

23~~5~~. The human *CYP2E1* gene exhibits several polymorphisms (for further information see the Human Cytochrome P450 Allele Nomenclature Database, available from: http://www.cypalleles.ki.se/cyp2e1.htm), some of which affect gene expression at the transcriptional level, whereas others affect enzyme activity. ~~(~~Pst I/Rsa polymorphism has three genotypes; wild type homozygous c1/c1; heterozygous c1/c2 and a rare homozygous form c2/c2~~)~~. ~~-~~Some published reviews suggest that certain polymorphic forms are associated with cancer susceptibility (Danko et al 2005; Trafalis et al 2010) and it is known that CYP2E1 polymorphisms impact on genotoxicity biomarkers induced by vinyl chloride in exposed workers (Dhillon et al 2011). However it is also reported that there is no association between CYP2E1 polymorphisms and cancer of the oesophagus (Yang et al 2005).

24~~6~~. Genetic polymorphisms have been investigated with a view to understanding the variation in susceptibility to alcohol related liver diseases. Lee et al (1997) concluded that there is a lack of association of the c1/c2, c2/c2 polymorphisms of CYP2E1 with the risk of hepatocellular carcinoma~~HCC~~ in humans. A meta-analysis of the association of CYP2E1 gene polymorphism and alcohol drinking on the risk of hepatocellular carcinoma~~HCC~~ showed that CYP2E1 Pst I/Rsa polymorphism was not associated with hepatocellular carcinoma~~HCC~~ risk, while the interaction between Pst I/Rsa polymorphism and alcohol consumption increased the risk of hepatocellular carcinoma~~HCC~~ (Liu et al 2014).

25~~7~~. Ishikawa et al (2006) examined the relationship between alcohol-drinking, ALDH2 and CYP2E1 polymorphisms and MN frequency in 248 Japanese men. The CYP2E1 classification genotypes were CYP2E1 *1/*1 (wild-type), *1/*3 or *3/*3. CYP2E1 *1/*1 genotype was associated with higher MN frequency in habitual drinkers and CYP2E1*2 was associated with lower MN. It should be noted that t~~[T~~hese are ~~a~~ different polymorphic variantsion to the c1/c1, c1/c2 classification above~~]~~.

Summary:

~~**Conclusions:**~~

26~~8~~. ~~It is~~ Several literature reviews have hypothesized that alcohol-induced oxidative stress is of importance in the pathogenesis of liver-induced injury, including carcinogenesis.

27~~9~~. Reactive oxygen species generated from oxidative metabolism or inflammatory processes give~~s~~ rise to lipid peroxidation products which in turn may yield mutagenic exocyclic DNA adducts.

~~30~~28. It is known that ethanol consumption results in the induction of CYP2E1, primarily in the liver, but also in certain extra-hepatic tissues such as the oesophagus and intestine. It is suggested that this induction enhances the metabolism of alcohol to acetaldehyde, the generation of ROS and the associated hazard of adduct formation. A correlation between CYP2E1 levels and DNA etheno adducts has been demonstrated in animal models and in humans. However an association between specific CYP2E1 polymorphisms and alcoholic liver disease ~~ALD/~~ and alcohol-induced carcinogenesis is not well defined. ~~appears to be weak.~~

Questions for Members:

- What are Members views on the hypothesis that CYP2E1 induction, ROS generation and etheno adduct formation are part of alcohol's mode of mutagenic and carcinogenic action?
- Do Members know of any other studies or information which they feel should be included in the alcohol mode of action discussions?
- Do Members feel that the topic has been covered in sufficient detail to write a section suitable for the statement on alcohol and mutagenicity?
- If sufficient, Members are invited to provide their comments and suggestions for the content of the section in the statement.

Secretariat
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