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Genotoxic activities of the food contaminant 5-hydroxymethylfurfural using different *in vitro* bioassays

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ABSTRACT

5-Hydroxymethylfurfural (5-HMF) is known as an indicator of quality deterioration in a wide range of foods. 5-HMF is formed as an intermediate in the Maillard reaction and has been identified in a wide variety of heat-processed foods. In recent years, the presence of 5-HMF in foods has raised toxicological concerns: data have shown cytotoxic, genotoxic and tumoral effects but further studies suggest that 5-HMF does not pose a serious health risk. However the subject is still a matter of debate. We investigated the genotoxicity of the food-borne contaminant 5-HMF using the Ames test, the micronucleus (MN) and the single-cell gel electrophoresis (SCGE) assays in the human metabolically active HepG2 cell line. Cytotoxic effect of 5-HMF was first assessed using Alamar Blue™ as a sensitive sub-lethal assay. 5-HMF did not induce any genic mutation in bacteria whatever the concentration in the Ames test. Furthermore, it does not induce clastogenic or aneugenic effects in the HepG2 cells. In contrast, 5-HMF induced HepG2 DNA damage at concentrations from 7.87 to 25 mM in the comet assay suggesting a weak genotoxic effect of 5-HMF in the HepG2 cells probably repaired.

1. Introduction

5-Hydroxymethylfurfural (5-HMF) (Fig. 1), a common major product of the Maillard reaction, is formed in many foods from reaction of reducing sugars and amino acids (Antal and Mok, 1990; Cuzzoni et al., 1988). Janzowski et al. (2000) reviewed the different available data regarding this compound. In an analytical screening of nearly 500 food samples, relatively high 5-HMF concentrations (exceeding 1 g/kg) have been reported for specific products such as dried fruits, caramel products, and juice made from dried plums. Very high peak concentrations up to 3.5 g/kg were found in dried pears, up to 9.5 g/kg in caramel products (Bachmann et al., 1997), up to 6.2 g/kg in instant coffee powder and up to 13.9 g/kg in respective substitutes (Schultheiss et al., 1999). Formation of 5-HMF in foods varies with processing and storage conditions and is especially dependent on temperature and pH. This has been confirmed in model experiments with fruit juices and concentrates as well as in heat-treated milk (Gomis et al., 1991; Kern, 1964; Morales et al., 1992). 5-HMF has also been identified in chewing tobacco, cigarette smoke and wood smoke (Baldwin et al., 1994; Black, 1966; Chou and Hee, 1994). A contribution of 5-HMF to flavour soy sauce has even been described (Kiahara, 1995).

It is not clear whether food-borne exposure to 5-HMF represents a potential health risk for humans. 5-HMF at high concentrations is cytotoxic, causing irritation to eyes, upper respiratory tract, skin and mucous membranes. An oral LD_{50} of 3.1 g/kg body weight has been determined in rats (Ulbricht et al., 1984). Neither data from epidemiologic studies or case reports on potential association of 5-HMF with cancer risk in humans nor chronic carcinogenicity studies are available. However, certain indications of 5-HMF for tumorigenic activities have been mentioned in rats and mice: 5-HMF can act as an initiator and also as a promotor, as shown for the induction of colonic aberrant crypt foci (Zhang et al., 1993). Induction of skin papillomas has been described by Surh and Tannenbaum (1994) after topical application of 10–25 μmol 5-HMF to mice. In contrast, in another study, the increase of skin tumour rates associated with 5-HMF-treatment was not statistically significant (Miyakawa et al., 1991).

Controversial results have been published on mutagenicity/genotoxicity of 5-HMF *in vitro*, particularly when tested with the

Abbreviations: AB, Alamar Blue™; B[*a*]P, benzo[a]pyrene; CYP, cytochrome P450; DMSO, dimethylsulfoxide; DMEM, Dulbecco's modified Eagle medium; FBS, foetal bovine serum; 5-HMF, 5-hydroxymethylfurfural; HPLC/MS, high performance liquid chromatography/mass spectrometry; IC50, concentration required for inducing 50% inhibition of the measured parameter; LD_{50}, lethal dose, 50% or median lethal dose.; MEM, minimum essential medium Eagle with EARL; BNC, binucleated cells; MN, micronuclei; OECD, Organisation for Economic Co-operation and Development; OTM, olive tail moment; RT-PCR, reverse transcription-polymerase chain reaction; SCGE, single-cell gel electrophoresis (or comet assay).

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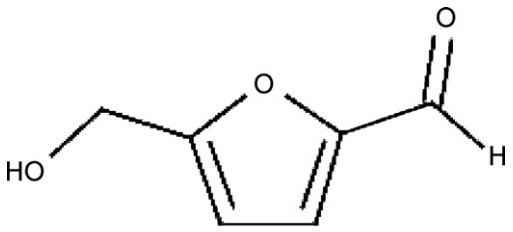

5-Hydroxymethyl-furfural (5-HMF)

CAS: 67-47-0

Fig. 1. Chemical structure and CAS number of 5-hydroxymethylfurfural (5-HMF).

Ames test. This test has often been performed on 2 strains only (TA 98–TA 100) instead of the 5 strains as recommended by the OECD guideline no. 471. The results varied also whether a metabolic activating system was used or not (Nishi et al., 1989). Furthermore, several authors (Surh and Tannenbaum, 1994; Lee et al., 1995; Sommer et al., 2003) have shown that the sulfotransferase system is required for detection of 5-HMF mutagenicity. These studies were performed under different conditions, then, a final conclusion concerning genotoxic/mutagenic activity of 5-HMF is still difficult. Considering the wide distribution and sometimes high concentrations of 5-HMF in certain foods, and also in fructose-containing solutions for intravenous injection, additional informations based on detailed dose–response experiments are required.

In this study, the Ames test was first performed according to the OECD guideline no. 471 to shed light on the mutagenicity of this food-borne compound using a prokaryotic model. To check the involvement of sulfotransferase in the toxic effect of 5-HMF, we chose a human cell line; HepG2; as *eukaryotic* model. Indeed, human cells expressing human xenobiotic metabolising enzymes (phases I, II and III) are more likely to be predictive of human susceptibility to the biological effects of chemicals, as RNA transcripts of cytochrome P450 (CYPs) and phase II enzymes, specially sulfotransferase system are present in HepG2 cells (Darroudi and Natarajan, 1993; Westerink and Schoonen, 2007a,b), this cell line is a reliable tool for toxicity assays. Indeed, the levels of CYP 1A1, 1A2, 2B6, 3A4 and SULT 1A1, 1A2, 1E1, 2A2 and 2A1 were mostly similar to levels found in primary human hepatocytes and this HepG2 cell line can be used to detect compounds giving false negative results in other *in vitro* assays (Knasmuller et al., 1998; Uhl et al., 2000; Lu et al., 2002).

Two genotoxic assays were performed on HepG2 cells: the micronucleus (MN) and the single-cell gel electrophoresis (SCGE) assays (Valentin-Severin et al., 2003) to study the mechanisms by which 5-HMF acts. The SCGE assay, also known as comet assay (McKelvey-Martin et al., 1993) was chosen to screen whether 5-HMF could affect HepG2 DNA. The MN assay was chosen as an indicator of both clastogenic and aneugenic effects (Kirsch-Volders et al., 2003), as this test is currently being incorporated into OECD guidelines for *in vitro* genotoxicity assays (OECD draft, Dec. 2007). In order to avoid false positive data in the genotoxicity assays, the Alamar Blue™ assay was selected as the complementary cytotoxicity test. It is a sub-lethal assay which is very sensitive and is useful for assessing cytotoxicity of agents within various chemical classes (Slaughter et al., 1999; O'Brien et al., 2000; Hamid et al., 2004).

2. Materials and methods

2.1. Chemicals, cells and medium

5-HMF [no. CAS 67-47-0] (purity 99%), benzo[*a*]pyrene (purity >99.9%), vinblastine sulphate (purity >96%), minimum essential medium (MEM), 100× non-essential aminoacids, dimethylsulfoxide (DMSO), propidium iodide, sodium sarcosinate, low melting point agarose, hydroxyaminomethane (Tris), acridine orange, benz[*a*]anthracene and cytochalasin B were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Heat-inactivated fetal bovine serum (FBS), L-glutamine, phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS), trypsin (0.05%)–EDTA (0.02%) were obtained from Invitrogen (Cergy-Pontoise, France). Normal melting-point agarose was purchased from Promega (Madison, USA). Alamar Blue™ was purchased from Biosource Europe (Nivelles, Belgique).

The HepG2 cell line was obtained from the ECACC (European Collection of Cell Culture, UK). The *Salmonella typhimurium* strains and S9 mix were kindly provided by UMR FLAVIC (INRA, Dijon, France).

2.2. Ames test

The Ames test was carried out using the plate incorporation method (with pre-incubation) with or without metabolic activation, with four histidine-dependent auxotrophic mutants of *S. typhimurium* strains, TA 98, TA 100, TA 1535, TA 1537, essentially as described by Maron and Ames (1983). As recommended by Brusick et al. (1980), a fifth strain was used: a tryptophan-dependent auxotrophic mutant *Escherichia coli* WP2*uvrapKM101*. The test strains were cultured in the liquid broth medium for 10 h at 37 °C under agitation. After incubation, 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) (the absence of metabolic activation) or 0.5 mL of S9 mix (the presence of metabolic activation), 0.1 mL of bacterial culture, and 50 μL of 5-HMF solutions (0.5 μg/mL up to 5000 μg/mL) were added to a test tube and pre-incubated 1 h at 37 °C. Two milliliters of semi-liquid superficial agar were added to the mixture and poured onto a minimal glucose agar plate. For the experiments with *S. typhimurium*, the top agar was supplemented with 10 mL of 0.5 mM histidine/biotin solution per 100 mL agar, and mutations to histidine independence were scored on minimal glucose agar plates. For experiments with *E. coli* strain, mutations to tryptophan independence were scored on minimal glucose agar plates supplemented with 10 mL of 0.5 mM tryptophan per 100 mL agar. The plates were incubated 48 h at 37 °C, and then the number of revertant colonies was counted. All experiments were carried out in triplicate using seven concentrations of 5-HMF. Mutagenic activities were expressed as induction factors, i.e. as multiples of the background levels.

2.3. HepG2 cell line

Routine monitoring has shown the HepG2 cells to be mycoplasma free (Mycoalert kit from Cambrex, Verviers, France). The cells were grown in monolayer culture in MEM supplemented with 2 mM L-glutamine, 1% non-essential aminoacids and 10% FBS in a humidified atmosphere of 5% CO_2 and at 37 °C. All cells used in the experiments were maintained and expanded in cell culture plastic flasks, (Falcon, Dutscher, France). Cells were divided every 7 days at 2.2×10^6 cells/75 cm^2 flask by trypsination. Stocks of cells were routinely frozen and stored in liquid N_2. Only cells from passages 8–17 were used in the experiments.

2.4. Seeding and treatment

For the Alamar Blue™ and comet assays, HepG2 cells were seeded into 96-well plate (Dutscher, France) in 200 μL of culture medium at a final concentration of 5×10^4 cells/well. For the micronucleus assay, individual wells of a 6-well plate (Falcon, Dutscher, France) were inoculated with 3 mL of the culture medium containing 7.5×10^6 HepG2 cells. After a 28-h incubation, the medium was removed and cells were exposed to the test substance for 20 h in 100 μL (comet and Alamar Blue™ assays) and in 2 mL (micronucleus test) of fresh complete MEM supplemented with only 0.5% FBS. 5-HMF, B[*a*]P (25 μM) and vinblastine sulfate (0.005 μM) were dissolved under sterile conditions in DMSO (purity >99%) such that the final concentration of DMSO in the medium was 0.25%. Control cells were exposed with medium containing only vehicle.

2.5. Alamar Blue™ cytotoxicity assay

The cell viability was performed using the Alamar Blue™ assay (AB) (O'Brien et al., 2000). AB is taken up in cells by passive diffusion and reduced in cytosol, mitochondria and/or microsomes. Continued growth maintains a reduced environment and causes the redox indicator to change from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form. After treatment, medium was removed and replaced by 100 μL AB/well, dissolved in MEM; yielding a final concentration of 10% AB. Plates were then placed into the incubator for 3 h. The absorbance was read on a plate reader (MRX Dynex) at 540 nm and 620 nm. The viability was expressed as a percentage of the control results (DMSO). GraphPad Prism® 4.0 software was used to calculate the concentrations associated with 50% inhibition of the measured parameter (IC_{50} values) using a Hill function non-linear regression analysis.

2.6. Alkaline comet assay

The comet assay, also known as the single-cell gel electrophoresis (SCGE) assay, is a very sensitive assay. The underlying principle is the ability of denatured DNA fragments to migrate during electrophoresis. Electrophoresis can be carried out under highly alkaline conditions (pH > 12.6) in order to detect single- and double-strand breaks and alkali-labile lesions. The assay was performed following the protocol of Singh et al. (1988) with some modifications for cell preparation