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CC/05/22

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

CARCINOGENICITY OF FURAN

Introduction

1. Furan is used industrially primarily as a solvent or in the synthesis of commercial compounds. It is also found in air pollution, tobacco smoke and many foods and beverages.
2. The EFSA Panel on contaminants in the food chain (CONTAM) issued a report on furan in food in December 2004. This report was produced in response to a US Food and Drug Administration (FDA) survey that revealed the occurrence of furan in a number of foods that undergo heat treatment, such as canned and jarred food and coffee, with levels ranging up to 125 µg/kg. The panel concluded that the available occurrence data were of an exploratory nature, and noted that a reliable risk assessment would require further data on both toxicity and exposure.
3. The CONTAM panel also concluded that the weight of evidence indicates furan-induced carcinogenicity is probably attributable to a genotoxic mechanism. They noted, however, that chronic toxicity with secondary cell proliferation may indirectly amplify the tumour response.
4. The COT reviewed the EFSA report in February, and made a recommendation for a systematic mode of action assessment in line with IPCS proposals. It was noted that the genotoxicity studies were relatively old, and the study designs may not be considered optimal by current standards. Members were also informed that additional data exist, in the form of a PhD thesis that was not available to the CONTAM panel, relating to possible mechanisms of carcinogenicity in rats treated with furan, including oxidative stress.
5. The COT concluded that it required further advice on the existing evidence for genotoxicity and carcinogenicity, and any needs for further research, from the COM and COC.

Advice from COM

6. COM was asked to advise on the mutagenicity of furan at its October meeting and, if appropriate, to recommend what further studies would be required to draw definite conclusions. The COM concluded that furan should be regarded as an *in vitro* mutagen but there was insufficient

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evidence to reach a conclusion on the available *in vivo* mutagenicity data. The draft COM minutes are appended at Annex 2.

Advice from COC

7. COC is asked to advise on the carcinogenicity of furan in light of the mode of action analysis provided and, if appropriate, to recommend what further studies would be required to draw definite conclusions.

Background

8. The critical toxicological effect of furan is carcinogenicity. In studies conducted by the National Toxicology Program (NTP; 1993), administration of furan in corn oil by gavage resulted in liver tumours and mononuclear cell leukaemia in rats, and liver tumours in mice. In 1995, IARC classified furan in group 2B, possibly carcinogenic to humans (IARC, 1995). The mechanism of furan carcinogenicity in rodents has not yet been fully elucidated, but both genotoxic and non-genotoxic mechanisms have been proposed.
9. A review of the toxicity, toxicokinetics, mutagenicity and a summary of the carcinogenicity studies of furan is attached at Annex 1. This review concentrates on furan and its key reactive metabolite, *cis*-2-butene-1,4-dial.

Hepatotoxicity

10. In a study conducted by the National Toxicology Program (NTP, 1993), F344 rats and B6C3F₁ mice were treated with furan for 2 years. Rats received 2, 4 or 8 mg/kg b.w. 5 days/week for up to 104 weeks. Mice received 0, 8 or 15 mg/kg b.w. A number of nonneoplastic liver lesions were observed. In rats, biliary tract fibrosis, hyperplasia and inflammation were seen at all doses tested, as well as hepatocellular cytomegaly, degeneration, hyperplasia, necrosis and vacuolisation (see annex 1, paragraph 46). Mice treated with both doses of furan developed biliary tract fibrosis, hyperplasia and inflammation, and hepatocellular cytomegaly, degeneration and necrosis.
11. Furan induced hepatotoxicity in the first 24 hours following administration in F344 rats (30 mg/kg b.w.) and B6C3F₁ mice (50 mg/kg b.w.; Wilson *et al.*, 1992). This was followed by an increase in cell proliferation 48 hours after treatment (see annex 1, paragraphs 47-48).
12. Incubation of furan with isolated rat hepatocytes resulted in cytolethality, as evidenced by a significant increase in lactate dehydrogenase leakage (Carfagna *et al.*, 1993; see annex 1, paragraph 49). This effect was prevented by adding the CYP inhibitor 1-phenylimidazole to the cultures, but increased in hepatocytes from rats that had been pre-treated with acetone.

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13. F344 rat hepatocytes treated with cytotoxic doses of furan show 95% loss of ATP due to uncoupling of hepatic oxidative phosphorylation, and similar effects are seen *in vivo* (Mugford *et al.*, 1997; see annex 1, paragraph 50).
14. Furan has also been shown to induce hepatic apoptosis in female B6C3F₁ mice, accompanied by an increase in hepatocyte proliferation (Fransson-Steen *et al.*, 1997). In this study, co-treatment with aminobenzotriazole, an irreversible CYP inhibitor, prevented furan-mediated hepatotoxicity (see annex 1, paragraph 51).

Carcinogenicity

15. Male B6C3F₁ mice given furan (8 or 15 mg/kg b.w.) by gavage 5 days per week for 104 weeks had a significantly increased incidence of hepatocellular adenomas and carcinomas compared with control mice. The incidence of benign pheochromocytomas of the adrenal gland was also increased in these animals (NTP, 1993; see annex 1, table 1). In females, hepatocellular adenomas were significantly increased with both doses of furan, while the incidence of hepatocellular carcinoma and benign pheochromocytomas of the adrenal gland was significantly increased only with the higher dose.
16. F344/N rats of each sex (n=70) were administered furan at 0, 2, 4, or 8 mg/kg b.w. by gavage 5 days per week for up to 104 weeks with interim kills of 10 rats per group after 9 and 15 months. These rats had a significantly increased incidence of cholangiocarcinoma compared with controls at all doses of furan tested (males: 0/50; 43/50; 48/50; 49/50; females: 0/50; 49/50; 50/50; 48/50) and was present in many rats of each sex at the 9- and 15-month interim evaluations (9-month: males - 0/10, 5/10, 7/10, 10/10; females - 0/10, 4/10, 9/10, 10/10; 15-month: males - 0/10, 7/10, 9/10, 6/10; females - 0/10, 9/10, 9/10, 7/10). The incidence of hepatocellular adenoma and mononuclear cell leukaemia was significantly increased in animals that received the two higher doses of furan (NTP, 1993). Hepatocellular carcinomas occurred only in male rats treated with 4 and 8 mg/kg b.w. furan (see annex 1, table 2).
17. In a separate study by Johansson *et al.* (1997), preweaning male B6C3F₁ mice were administered furan as either a single dose (400 mg/kg b.w.) or as six multiple doses (200 mg/kg b.w.). Animals in the single-dose group developed a statistically non-significant increase in hepatic adenomas and carcinomas compared with controls. In the multiple-dose group, there was a significantly increased incidence of hepatocellular neoplasms compared with controls and in comparison with the single-dose group. In this investigation, no histopathological evidence of chronic liver cytotoxicity was observed (see annex 1, paragraph 53).
18. Male F344 rats given furan (30 mg/kg b.w.) by gavage five days per week for 9, 12 and 13 weeks had a high incidence of hepatic adenocarcinoma at

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16 months after initiation of chemical exposure (Elmore and Sirica, 1993; see annex 1, paragraph 57). The majority of the furan-induced hepatic adenocarcinomas examined showed evidence of small intestinal differentiation.

19. In a separate 2-year study (NTP, 1993), 50 male F344/N rats received 30 mg/kg furan in corn oil by gavage 5 days/week for 13 weeks and were then maintained for the remainder of the 2 years without additional furan administration. Groups of 10 animals were evaluated at the end of the furan administration period, and again at 9 months and 15 months. Cholangiocarcinoma of the liver occurred with an overall incidence of 100 % (40/40) and hepatocellular carcinoma occurred with an overall incidence of 15 % (6/40) in all rats that survived at least 9 months. Hepatocellular carcinoma was first observed in 2 males at the 15-month interim evaluation. No details on statistics are provided with this study. This study used a single dose level and no specific control group is described in the NTP report.

Mode of action.

20. COM concluded that furan should be regarded as an *in vitro* mutagen but there was insufficient evidence to reach a conclusion on the available *in vivo* mutagenicity data.
21. At least two possible modes of action for furan induced liver changes have been described, altered cell proliferation and oxidative stress mechanisms. It is possible that there could be different mechanisms associated with different tumours reported.

Information on altered cell proliferation.

22. Butterworth and co-workers (1994) examined expression profiles for *myc*, *fos* and *Ha-ras* oncogenes. Rats received either single gavage exposure to 30 mg/kg bw furan in male F344/CrlBR rats (n=4 for those killed 0.5 to 12 hours post treatment or n=5 for those killed 12 hours to 8 days post treatment) or repeat exposure to 8 mg/kg bw furan for up to 6 weeks (5 days/week) in male and female F344/CrlBR rats (n=6). Male B6C3F1/CrlBR mice were also dosed with 15 mg/kg bw furan for up to 6 weeks (5 days/week). Following single exposure *myc*, *fos* and *Ha-ras* all showed increases in expression peaking at 12 to 24 hours post-exposure. However following repeat exposure changes were only observed in *myc* expression. These increases were much more pronounced in male than female rats both in timing (occurring at all time points compared with only at 6 weeks) and magnitude (15 fold compared with 3 fold at 6 weeks). No increases were seen in mice. (The full paper is appended at Annex 3)
23. A series of studies on expression of c-neu, c-met, hepatocyte growth factor/scatter factor (HGF/SF) and CDX1 in F344 rats (Sirica et al. 1997, 1999, 2000a & 2000b). The authors hypothesis was that the early formed

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metaplastic lesions associated with cholangiofibrosis represented a precancerous pool of initiated cells directly related to subsequent development of a primary hepatic intestinal-type of cholangiosarcoma rather than simply being a reactive change. This was supported by a series of cumulative findings

- neoplastic glands of primary intestinal-type tumours induced by furan in rat liver exhibit evidence of goblet cell, Paneth cell and serotonin positive neuroendocrine cell differentiation. These are similar to earlier appearing intestinal metaplastic glands in putative precancerous hepatic cholangiofibrotic tissue
- CDX1 expression is a prominent feature of mucin-producing neoplastic glands of furan-induced primary hepatic tumours
- intestinal metaplastic glands in hepatic cholangiofibrotic tissue and neoplastic glands in subsequently developed primary hepatic intestinal-type of cholangiosarcoma exhibit proliferating cell nuclear antigen-labelling indices three to four-fold higher than in hyperplastic biliary ducts and ductules induced by furan in rat liver
- intestinal metaplastic glands in hepatic cholangiofibrotic tissue and neoplastic glands are characterised by over-expression of c-Met and c-Neu receptor tyrosine kinases

24. The concomitant over expression of c-met and HGF/SF which were considered characteristic of the induction of putative precancerous and cancerous biliary epithelial cells following furan treatment, appear following 6 weeks of furan treatment whilst CDX1 appears following 3 weeks of furan treatment. The dose level and duration of dosing in the initial studies from which tissues were obtained for expression studies on c-neu and c-met are unclear. The references cited as the source of liver tissues involved a range of furan doses between 30 and 45 mg/kg bw and durations of 2 to 19 weeks. (The full papers are appended at Annex 4)

Unpublished studies.

25. Following the COT discussions, a COT member after contacting the authors provided the Secretariat with an unpublished manuscript examining the dose response relationship between tumour proliferation and cell death with compensatory proliferation in the target organ (Annex 5). The study involved dosing groups of female B6C3F1 mice for 3 weeks (n=15) or 2 years with 0, 0.5, 1, 2, 4 and 8 mg/kg bw furan (n=100 at 0.5, n=75 at 1 and n=50 at 2 and above). After 3 weeks there were dose dependent increases in hepatic cytotoxicity and ALT (statistically significant at 1 and above). Hepatocyte proliferation was significantly increased at 8 mg/kg bw but there was a trend of increasing numbers of mice with increased mitotic figures from 1 mg/kg bw upwards. After 2 years absolute and relative liver weights and incidence of liver nodules were increased at doses above 4 mg/kg bw. There was a dose dependent increase in the incidence and severity of hepatic cytotoxicity which were statistically significant at doses above 1 mg/kg bw

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Information on oxidative stress mechanisms.

26. During the COT discussions the Secretariat was informed of two recent theses at Birmingham University indicating that furan induced tumours may result from oxidative stress mechanisms. Both theses have now been examined but the results are not yet published. The research group has been approached and a representative will present their unpublished findings to the Committee. An abstract has been requested and will be circulated as a separate annex once received. As the Secretariat has not yet seen these data it is not possible to incorporate them into the analysis at this stage and their role is an additional uncertainty.

Analysis

27. Furan should be regarded as an *in vitro* mutagen but there was insufficient evidence to reach a conclusion on its genotoxicity *in vivo* on the available *in vivo* mutagenicity data. There are a number of compounds for which tumour formation in rodents is preceded by cytotoxicity. Following furan exposure a number of changes in gene expression occur leading to altered cell proliferation. The initial steps leading to these alterations in gene expression and the role of oxidative stress are unclear. The key events are hepatic cytotoxicity and regenerative cell proliferation which result in cholangiofibrosis and tumour formation. There is evidence for a dose response relationship between cytotoxicity and subsequent liver tumour formation.

Advice required from the COC

28. The COC are asked to advise on the following points.

- i) Is a threshold approach for furan induced tumours appropriate?
- ii) Does sufficient evidence exist to permit a mode of action analysis of carcinogenicity resulting from furan and/or its metabolites?
- iii) What further research, if any, would help to determine whether assumption of a threshold mode of action is appropriate?

Food Standards Agency
November 2005

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Radaeva S, Ferreira-Gonzalez A & Sirica A E; Overexpression of C-NEU and C-MET during rat liver cholangiocarcinogenesis: a link between biliary intestinal metaplasia and mucin-producing cholangiosarcoma. *Hepatology* 29:1453-1462 (1999)

Ren P, Silberg D G & Sirica A E; Expression of an intestine-specific transcription factor (CDX1) in intestinal metaplasia and in subsequently developed intestinal type of cholangiosarcoma in rat liver. *Am J Pathol* 156:621-6274 (2000)a

Sirica A E, Radaeva S & Caran N; NEU overexpression in the furan rat model of cholangiocarcinogenesis compared with biliary ductal cell hyperplasia. *Am J Pathol* 151:1685-1694 (1997)

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ANNEX 1 TO CC/05/22

FURAN

Chemistry

1. Furan is an aromatic molecule that consists of a five-membered ring containing two double bonds and an oxygen atom. It is the parent structure of a large group of naturally occurring compounds, including furfural and furfuryl alcohol (Burka *et al.*, 1991). Furan is a colourless, volatile organic liquid with a boiling point of 31.4°C, and is insoluble in water, but soluble in acetone, benzene, diethyl ether and ethanol.
2. The introduction of substituents into the furan ring may significantly alter its reactivity. Strong electron-withdrawing substituents such as NO₂ make the furan ring more resistant to oxidation, while alkyl substituents may enhance oxidation (NTP, 1993).
3. Furan serves as an intermediate in the synthesis and preparation of many linear polymers, used in the production of pharmaceuticals, agricultural chemicals and stabilisers, and as a solvent for resins and lacquers.
4. Furan has also been detected as an environmental contaminant in smog, tobacco smoke and many foods and beverages (Capurro, 1973; Maga *et al.*, 1979; FDA, 2004). The primary route of formation of furan in food has been reported to be through the thermal decomposition of organic compounds, particularly carbohydrates (Maga, 1979). Furan has been detected following thermal degradation of glucose, glyceraldehydes, D-erythrose, pentosans, hexoses and polysaccharide, and in a lactose-casein browning system (Maga, 1979). In addition, thermal oxidation of lipid and decomposition of ascorbic acid derivatives are also possible routes of furan production. In view of the wide variety of foods in which furan has been detected, such as roasted coffee, canned meat and various vegetables, it is probable that there are multiple routes of formation.

Absorption, distribution, metabolism and excretion

Absorption

5. Disposition studies in male F344 rats given [2,5-¹⁴C]furan by gavage indicate that furan is rapidly and extensively absorbed following oral administration (Burka *et al.*, 1991). Furan is also absorbed following inhalation. A physiologically based pharmacokinetic (PBPK) model

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simulating inhalation exposure to 10 ppm furan for 4 hours indicated that the absorbed dose was approximately between 3- and 10-fold less in humans (0.4 mg/kg b.w.) than in rats (1.4 mg/kg b.w.) or mice (4.1 mg/kg b.w.), respectively (Kedderis and Held, 1996). These results indicate that target organ concentration, rather than external exposure, might be most appropriate for interspecies comparison of dose. A further study using a PBPK model indicated that the blood concentration of furan following inhalation exposure is likely to be greater in children than adults, by a factor of 1.5 (Price *et al.*, 2003).

Distribution

6. Twenty-four hours after administration of a single dose of [2,5-¹⁴C]furan (8 mg/kg b.w.) by gavage to F344 rats, 19% of the administered radioactivity remained in the tissues (Burka *et al.*, 1991; see annex 8). Of this, 13% was found in the liver, while quantities of less than 1% were detected in the kidney, large intestine, small intestine, stomach, forestomach, blood and lung.
7. In the same study, repeated daily dosing of furan (8 mg/kg b.w.) resulted in an increase in furan-derived radioactivity in liver, kidney and blood. This increase was linear in kidney and blood during the first 4 days, but non-linear by 8 days. In liver, the increase in furan radioactivity was non-linear by day 4. Following 8 daily doses, the concentration of furan-derived radioactivity increased four-fold in the liver, and by approximately six-fold in kidney and blood.

Metabolism

8. Although information exists describing the metabolism of compounds containing the furan ring, the metabolism of furan itself is less well characterised.
9. Furan is extensively metabolised *in vivo*, and a PBPK model for furan in rats developed by Kedderis *et al.* (1993) predicted 84% metabolism and 16% exhalation of the parent compound following a single oral dose of 8 mg/kg b.w.. In the disposition studies performed in F344 rats by Burka *et al.* (1991), carbon dioxide was identified as a major metabolite, indicating ring opening followed by carbon oxidation. In addition, at least 10 unidentified metabolites were detected in the urine by high performance liquid chromatography (HPLC). *cis*-2-Butene-1,4-dial has subsequently been postulated to be the key reactive metabolite of furan (Chen *et al.*, 1995; see figure 1). It has been proposed that a transient epoxide may be an intermediate in the formation of *cis*-2-butene-1,4-dial, although this was not observed by Chen *et al.*

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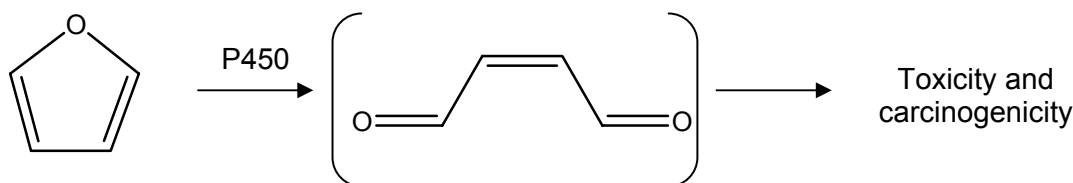


Figure 1. Proposed bioactivation pathway for Furan (Byrns et al., 2004)

10. Cytochrome P450 (CYP) enzymes are involved in furan metabolism. Metabolism of furan *in vivo* is inhibited by pyrazole, a CYP inhibitor (Kedderis *et al.*, 1993), while furan metabolism *in vitro* is increased five-fold in hepatocytes isolated from rats treated with acetone, but not from rats treated with phenobarbital, indicating that CYP2E1 is a major catalyst of furan metabolism.
11. A study using isolated hepatocytes demonstrated that human cells rapidly metabolise furan (Kedderis and Held, 1996). Isolated hepatocytes from mice, rats and humans rapidly metabolised furan with V_{\max} values of 48, 18 and 19-44 nmol/hour/ 10^6 hepatocytes, respectively. K_M values in mice, rats and humans were 1.0, 0.4 and 2.1-3.3 μM , respectively. Further analysis using physiologically based dosimetry models indicated that initial rates of furan oxidation in rat, mouse and human liver were approximately 13-, 24- and 37-fold greater than the respective rates of blood flow delivery of furan to the liver. The researchers concluded that bioactivation of furan is limited by hepatic blood flow, and is unlikely to be affected by the induction of CYP2E1 or interindividual variations in CYP2E1 activity among humans.
12. There is little information regarding phase II metabolism of furan. However, *cis*-2-butene-1,4-dial has been shown to react readily with glutathione (GSH) and amino acids (Chen *et al.*, 1997). Covalent binding of reactive furan metabolites to microsomal protein *in vitro* is inhibited by GSH and, to a lesser extent, semicarbazide, (Parmar and Burka, 1993).

Elimination

13. Within the first 24 hours of a single oral dose of [2,5- ^{14}C]furan to male F344 rats, approximately 80% of the radioactivity was eliminated via the lung, urine and faeces, while 19% remained in the tissues (Burka *et al.*, 1991). Around 14% of the administered dose was expired as unchanged furan and 26% as carbon dioxide. Radioactivity in urine and faeces accounted for 20 and 22% of the administered dose, respectively.

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14. Over 8 days following furan administration, elimination of radioactivity from the liver appeared to follow first-order kinetics with a half-life of 1.8 days. The kinetics for elimination from kidney and blood were more complex, although details are not provided. The blood concentration of radioactivity remained more or less constant during the 8 days following a single dose.
15. The effect of multiple daily dosing on elimination of furan was also investigated in this study. The percentage of total administered furan eliminated in the faeces was the same for 8 daily doses as for one single dose followed for 8 days. In contrast, the percentage of administered reactivity eliminated via the urine showed an upward trend after 4 days in the multiple-dose experiment. Following 8 daily doses, 33% of the radioactivity had been eliminated in the urine, compared with 20% in the single-dose experiment.

Genotoxicity

Bacterial tests for gene mutation

16. Furan was tested for mutagenicity in the *Salmonella typhimurium* strains TA100, TA1535, TA1537 and TA98, using a preincubation protocol in the presence and absence of Aroclor 1254-induced rat or hamster S9 mix (NTP, 1993; see annex 2, pp248, 251 and table E1). Each study included at least five doses of furan, ranging from 33 to 10,000 µg/plate, as well as positive and negative controls. Studies were performed in triplicate assays, and repeated. Mutagenic activity was not seen in any of the strain/activation combinations. A precipitate was observed in several of the trials at concentrations of 1,000 µg/plate or higher. Since furan is a liquid, the nature of this precipitate is unclear, and no further details are provided in the NTP report.
17. In another *S. typhimurium* study, furan was assessed in strains TA98 and TA100 (Lee *et al.*, 1994; see annex 3). A standard plate-incorporation method was used, in the presence and absence of Aroclor 1254-induced rat liver S9 mix. Experiments were performed in triplicate plates at least twice. At concentrations ranging from 0.8-100 µmole/plate (calculated as equivalent to 54-6,807 µg/plate), furan did not show mutagenic activity in strain TA98. In strain TA100, however, furan was reported as having weak mutagenic activity with and without S9. Of note, the positive control in the TA100 studies without S9 (dinitropyrene isomers) produced only a 1.2-fold increase in the number of revertants over the negative control. Information on toxicity is not provided in this paper.
18. Peterson *et al.* (2000; see annex 4) examined the genotoxic activity of *cis*-2-butene-1,4-dial in several strains of *S. typhimurium*. In two experiments consisting of four replicates, *cis*-2-butene-1,4-dial at concentrations ranging from 1.4-2.1 µmol/plate (calculated as equivalent to 118-177 µg/plate) was mutagenic in TA104, a strain sensitive to aldehydes.

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Concentrations of 2.9 and 4.3 $\mu\text{mol}/\text{plate}$ (calculated as equivalent to 244 and 362 $\mu\text{g}/\text{plate}$) were reported to be toxic. *cis*-2-Butene-1,4-dial was not mutagenic in strains TA97, TA98, TA100 and TA102, however. Preliminary experiments showed that addition of glutathione during the post-incubation period reduced the cytotoxic effects of *cis*-2-butene-1,4-dial. In addition, the cytotoxic and genotoxic activity of the metabolite was prevented by incubation of *cis*-2-butene-1,4-dial with glutathione prior to the addition of cells. In comparison with other reactive aldehydes, the mutagenic activity of *cis*-2-butene-1,4-dial was reported to be similar to that of crotonaldehyde, acrolein and glyoxal.

In vitro tests for clastogenicity

19. Furan was tested for induction of sister chromatid exchange (SCE) and chromosomal aberrations (CA) in Chinese Hamster Ovary (CHO) cells (NTP, 1993; see annex 2 p249, 251 and tables E3 and E4). For both SCE and CA, two trials were conducted in the absence of Aroclor 1254-induced rat liver S9, and one trial was performed in the presence of S9. Each test consisted of solvent and positive controls, and at least three doses of furan. For the SCE test, 50 second-division metaphase cells were scored for frequency of SCE at each dose level. For the CA test, 200 first-division metaphase cells were scored at each dose level. No details on cytotoxicity are provided for these studies.
20. Exposure to furan for 26 hours at concentrations of 1.6-160 $\mu\text{g}/\text{ml}$ resulted in a positive SCE response in one of the trials conducted in the absence of S9, with a concentration-related 21.97-48.49% increase in SCEs/chromosome over control. However, only 6 cells were counted at the highest concentration in this trial. In the second trial, a weak positive result was seen, with a significant, 27.77% increase in the percentage of SCEs/chromosome over control seen only with the highest concentration of furan used (160 $\mu\text{g}/\text{ml}$). A third trial, involving incubation with furan in the presence of S9 for 2 hours, was also classified as a weak positive, with a 36.77% increase in SCE/chromosome at the highest concentration of furan tested (500 $\mu\text{g}/\text{ml}$).
21. In the CA study in the absence of S9, cells were incubated with furan for 8-10 hours. For the test with S9, cells were treated for 2 hours. Furan exposure resulted in positive responses at concentrations ranging from 100-500 $\mu\text{g}/\text{ml}$ in the two studies performed without S9, and at concentrations of 500 and 1,000 $\mu\text{g}/\text{ml}$ in the study performed with S9.
22. In another study using CHO cells (Stich *et al.*, 1981), the effect of furan treatment on chromatid breaks and chromatid exchanges was examined. Cells were treated with and without Aroclor 1254-induced rat liver S9 mix for 3 hours, and sampled 20 hours after the completion of treatment. Colchicine was added to the cells 4 hours prior to sampling. In the absence of S9 activation, furan did not show clastogenic activity. In the presence of S9, however, furan treatment resulted in a dose-dependent

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increase in chromatid breaks and chromatid exchanges. The exact dose range is not given, but in a graph appears to range from around 25-250 mM. Specific figures are given only for 184 mM furan. At this concentration, 0.03 chromatid breaks and 0.31 chromatid exchanges per diploid metaphase cell were observed, in comparison with 0 breaks or exchanges in control cells. Aflatoxin B1 (up to 0.005 mM) was used as a positive control for the activity of the S9 mixture, but results are only presented in graphical form. In the graphs, the percentage of metaphase plates with CAs appears to be between 20-30% at the highest concentration of furan used, compared with almost 80% at the highest concentration of aflatoxin B1. No information on cytotoxicity in the presence of furan is provided in this report.

23. In an abstract, Mugford and Kedderis (1996) reported that treatment with 100 μ M furan induced DNA double strand breaks in hepatocytes isolated from male F-344 rats. In this study, cells were incubated with furan for 0-4 hours at 37°C. DNA double strand breaks were detected following 30 minutes of furan exposure, and continued to be observed at all subsequent time points studied. In a second abstract, it was reported that the addition of 1-phenylimidazole to the incubations reduced the formation of furan-mediated DNA double strand breaks indicating that cytochrome P450 metabolism was required (Mugford and Kedderis, 1997). The endonuclease inhibitor aurintricarboxylic acid also reduced the formation of furan-mediated DNA double strand breaks.
24. In a recent study, Glatt *et al.* (2005) tested furan dissolved in dimethylsulphoxide for induction of SCE in a Chinese hamster V79 cell line engineered to express human cytochrome P450 (CYP) 2E1 – the major enzyme involved in furan biotransformation – and human sulphotransferase (SULT) 1A1. Furan treatment resulted in a statistically significant increase in SCE at a range of concentrations between 3 and 16,000 μ M. The concentration-response curve was unusual, however. The increase in SCE compared with control cells remained relatively constant over all concentrations tested, ranging from 4.00 per metaphase at 3 μ M to 5.89 at 16,000 μ M. The cell proliferation index was reduced in cells treated with all concentrations of furan compared with control cells. A similar, although slightly weaker, response was seen in the parent V79-MZ cell line at concentrations ranging from 1,000-16,000 μ M.
25. CHO-K1 cells labelled with 14 C were exposed to the furan metabolite *cis*-2-butene-1,4-dial for 90 minutes at 37°C (Marinari *et al.*, 1984; see annex 5). Alkaline elution assays were performed on the cells both with and without exposure to 1 mM methyl methanesulphonate (MMS). The assay is based on the principle that single-strand breaks increase elution in the assay performed without MMS, while cross-links decrease elution in the assay performed with MMS. *cis*-2-Butene-1,4-dial induced DNA single strand breaks at concentrations of 0.17, 0.5 and 1.5 mM, and DNA cross links at 1.5 mM. Cell viability at these concentrations was 99.8, 98.7 and 79.7% of control, respectively.

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Mammalian cell mutation assays

26. Furan has been tested in the absence of S9 for mutation at the thymidine kinase locus of L5178Y mouse lymphoma cells (NTP, 1993; see annex 2 p 248, 251 and table E2). The trial was performed three times in duplicate. A negative response was seen in the first trial, with concentrations of furan up to 2,000 µg/ml. In a second trial, however, furan produced a significant concentration-related mutagenic response at concentrations of 2,600, 3,200 and 3,800 µg/ml. Relative total growth was $\leq 52\%$ at these concentrations, and below 10% in the cultures treated with 3,800 µg/ml. In the third trial, furan evoked a concentration-dependent mutagenic response at five concentrations ranging from 1,139 µg/ml to 3,090 µg/ml. Relative total growth was below 50% at the two highest concentrations (2,604 and 3,090 µg/ml). On the basis of these results, the authors concluded that furan was mutagenic in this assay.

In vivo assays for clastogenicity

27. As part of the NTP study (1993), *in vivo* bone marrow cell SCE and CA tests were performed in male B6C3F₁ mice (see annex 2, pp250-252 and tables E6 and E7).

28. Furan was tested for induction of SCEs using two protocols. The first experiment used a standard harvest time of 23 hours, with a dose range of 87.5-350 mg/kg b.w. furan. The second had a delayed harvest of 42 hours, and a dose range of 25-100 mg/kg b.w.. Mice were injected intraperitoneally with furan dissolved in corn oil, corn oil alone, or 100 mg/kg b.w. dimethylbenzanthracene as a positive control. In total, 25 second-division metaphase cells were scored from each of four animals per treatment. In both the standard and delayed harvest protocols, no increase in SCEs was observed in bone marrow cells of mice treated with furan.

29. Two protocols were also used for the CA test: a protocol with a standard harvest time of 17 hours and a dose range of 87.5-350 mg/kg b.w. furan, and a second with a delayed harvest of 36 hours and a dose range of 62.5-250 mg/kg b.w.. The same positive and negative controls were used as in the SCE test. For the CA studies, 50 first-division metaphase cells were scored from each of eight animals per treatment. No increase in CAs was seen in B6C3F₁ mice treated with furan under the standard harvest protocol.

30. However, two trials performed with the extended harvest protocol detected an increase in the percentage of cells with CA at the highest dose tested, 250 mg/kg b.w., but not at the two lower doses of 62.5 and 125 mg/kg b.w.. In these two studies, furan at 250 mg/kg b.w. resulted in 12-fold and 11-fold increases in the percentage of cells with CA, compared with 19-fold and 10-fold increases, respectively, with the positive control,

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dimethylbenzanthracene. No information on toxicity in the experimental animals is provided in the NTP report, although it is noted that a dose range finding study was initially performed in the absence of adequate toxicity information from the literature. As a result of this study, the highest dose of furan was limited to 350 mg/kg b.w.. In a study by Wilson *et al.* (1992), however, moderate midzonal degeneration and necrosis was seen in liver sections from male B6C3F₁ mice that had received a single dose of 50 mg/kg b.w. furan by gavage 12 and 24 hours previously (see paragraphs 47-48 and annex 7). This was followed by cell proliferation 48 hours after treatment. Wilson *et al.* also noted that the dose of 250 mg/kg causes extensive liver necrosis, and is at the limit of lethality to the animal. COM guidance states that positive *in vivo* mutagenicity data in bone marrow assays at dose levels that are associated with high levels of toxicity or lethality cannot be interpreted with certainty because of the confounding effects of toxicity (COM, 2003).

Unscheduled DNA synthesis

31. Wilson *et al.* (1992) assayed for unscheduled DNA synthesis (UDS) using the *in vivo* hepatocyte repair assay (see annex 7). Male F344 rats and male B6C3F₁ mice were administered furan in corn oil by gavage, and hepatocytes were isolated 2 or 12 hours later. In general, 25 cells per slide, three slides per animal and three animals were analysed for each dose. Negative and positive controls were also administered. No increase in UDS was seen in hepatocytes isolated from mice following exposure to furan at concentrations ranging from 10-200 mg/kg b.w., or in hepatocytes from rats given 5-100 mg/kg b.w. furan. The percentage of cells with ≥ 5 nuclear grains (NG), viewed as a positive response in this study, was less than 5% in mouse hepatocytes exposed *in vivo* to furan at any of the doses used, at either time point. With the positive control (dimethylnitrosamine [DMN]), over 90% of cells had ≥ 5 NG at both time points. In rat hepatocytes, furan treatment *in vivo* resulted in less than 5% of cells with ≥ 5 NG at either time point, in comparison with 100% of cells exposed to DMN (20 mg/kg) for 2 hours, and 57% of cells exposed to 2-acetylaminofluorene (50 mg/kg) for 12 hours.

Assay for covalent binding to DNA

32. Radiolabelled furan ([2,5-¹⁴C]furan, 8 mg/kg b.w.) was administered to male F344 rats for a disposition study (Burka *et al.*, 1991; see annex 8). The specific activity of the [2,5-¹⁴C]furan was 56 mCi/mmol, and this was diluted with nonlabelled furan prior to administration in order to deliver approximately 10 μ Ci/kg b.w. (specific activity estimated as 75 μ Ci/mmol [Peterson *et al.*, 2000]). Animals (three for each time-point) received either a single dose or 8 daily doses of furan dissolved in corn oil (8 mg/kg body weight) by oral gavage, and were sacrificed at time points ranging from 1-8 days after initial dosing.

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33. In total, 19% of the administered radioactivity remained in the tissues 24 hours after a single dose, with the majority (13%) found in the liver. Only 20% of the radioactivity in the liver was extractable by organic solvent. Following extraction of liver homogenate with phenol-chloroform-amyl alcohol, most of the radioactivity was in the protein interlayer. While there was some radioactivity in the DNA-containing aqueous layer, this was lost during subsequent processing with RNase, protease and reprecipitation. In contrast, radioactivity in the protein layer was not lost upon further extraction with organic solvents. Repeated daily dosing resulted in an approximately linear increase in covalent binding to liver protein over the first 4 days.
34. In an attempt to obtain higher levels of DNA-bound radioactivity, two rats were dosed intraperitoneally with 8 mg/kg b.w. undiluted [2,5-¹⁴C]furan. In this study, radioactivity associated with the DNA layer was again lost upon further processing. The researchers therefore concluded that there was either no binding to DNA or the furan-DNA adduct was not stable during the isolation procedure.

Other genotoxicity studies

Cell free systems

35. *cis*-2-Butene-1,4-dial reacts with 2'-deoxycytidine (dCyd), 2'-deoxyribo-guanosine (dGuo) and 2'-deoxyadenosine (dAdo) to form adducts (Gingipalli and Dedon, 2001; Byrns *et al.*, 2002). In these studies, *cis*-2-butene-1,4-dial did not react with thymidine. HPLC analysis indicated that the rate of reaction with deoxyribonucleosides was dependent on pH (Byrns *et al.*, 2002). At pH 6.5, the relative reactivity was dCyd > dGuo > dAdo, while at pH 8.0 it was dGuo > dCyd > dAdo. The initially formed dCyd adducts are relatively stable, while the dAdo and dGuo adducts are unstable and rearrange to form secondary products. Subsequent analysis with UV absorbance, fluorescence, ¹H NMR and mass spectral data indicated that the primary dAdo and dGuo adducts undergo dehydration to form substituted etheno products (Byrns *et al.*, 2004). The authors of this study noted that these secondary adducts retained a reactive aldehyde with the potential to form DNA cross-links.

Drosophila

36. Furan was tested for mutagenic activity in germ cells of male *Drosophila melanogaster* using the sex-linked recessive lethal (SLRL) assay (NTP, 1993; see annex 2, pp250-251, and table E5). Adult Canton-S males were subjected to feeding or injection exposure at a level shown by preliminary toxicity tests to result in approximately 30% mortality following 72 hours of

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feeding or 24 hours after injection. This was 10,000 ppm in the feeding studies, and 25,000 ppm in the injection experiments.

37. In total, over 5,000 control chromosomes and 5,000 treated chromosomes were scored for both feeding and injection studies. Furan did not show mutagenic activity in either experiment.

Mutation of proto-oncogenes in furan-induced liver tumours

38. Reynolds *et al.* (1987) transfected DNA from hepatocellular adenomas and carcinomas arising in vehicle or furan-treated B6C3F₁ mice from the NTP 2-year bioassay into NIH3T3 cells (see annex 9). They examined resulting foci for the presence of activated oncogenes, and the activating mutation. In tumours from control mice, 3/3 adenomas and 12/14 carcinomas had a transforming H-*ras* oncogene with a point mutation in codon 61. In addition, an activated *raf* gene was identified in one carcinoma. Although 10/13 tumours from furan-treated mice had a transforming H-*ras* oncogene, the spectrum of mutations in the furan-induced tumours was different to that of the control tumours. While four adenomas and one carcinoma had a point mutation at codon 61, three adenomas and one carcinoma had a point mutation at codon 117 (the mutation in the other carcinoma was not reported). In the remaining furan-induced tumours, an activated K-*ras* oncogene or an activated *raf* oncogene was identified. The different spectrum of mutations in the furan-induced tumours compared with that seen in the spontaneous tumours led the researchers to hypothesise that furan is mutagenic.

39. Prewaning male B6C3F₁ mice were administered furan, either as a single dose of 400 mg/kg b.w. i.p. or as six doses of 200 mg/kg b.w. i.p. (Johansson *et al.*, 1997; see annex 10) The relative frequency of H-*ras*-1 activation was 82% in tumours analysed from the single-dose group, and 32% in tumours analysed from the multiple-dose group. The frequency of H-*ras*-1 activation in the respective control animals was 33% and 58%. In total, 28 tumours were analysed for each treatment group, except the single-dose control, where just three tumours were assessed. Noting that the 82% relative frequency of H-*ras*-1 activation in the tumours from the single-dose study was greater than that of the controls or historical control frequencies (54%), the authors postulated that furan-induced hepatocarcinogenicity may be at least partially attributable to *in vivo* genotoxicity.

Acute Toxicity

40. Egle and Gochberg (1979) evaluated the acute toxicity of furan in male Swiss mice and Sprague-Dawley rats. Rats were injected intraperitoneally with furan (dose range 1.5-10 mg/kg b.w.) in a solution of benzyl alcohol, citric acid, Tween 80 and deionised water. Mice were exposed to furan either by i.p. injection (dose range 5-20 mg/kg b.w.) or by inhalation (concentration range 0.03-1 µg/ml) for 1 hour. The LD₅₀ for furan in

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solution was reported to be 5.2 mg/kg in rats, and 6.94 mg/kg in mice. No deaths were seen in control animals injected with the same solution without furan. In the mouse inhalation studies, the LC₅₀ for furan was found to be 0.12 µg/ml. Examination of the lungs of these animals revealed inflammation and fluid accumulation.

41. A single intraperitoneal injection with furan dissolved in sesame oil (300 and 340 mg/kg) resulted in hepatic and renal necrosis in adult male Swiss albino mice (McMurtry and Mitchell, 1977). Furan was reported to produce midzonal-centrilobular necrosis of parenchymal hepatocytes at smaller doses, and centrilobular lesions at larger doses, although further details are not provided. Pretreatment with the CYP inhibitor piperonyl butoxide decreased the incidence and severity of hepatic and renal necrosis. An anecdotal comment in this report states that furan was also seen to be a potent pulmonary toxin.
42. Gammal *et al.* (1984) showed that intraperitoneal injections of furan in mice produced bronchiolar necrosis.

Hepatotoxicity

43. Furan was evaluated in the National Toxicology Program (NTP, 1993) by administration in corn oil by gavage to F344/N rats and B6C3F₁ mice of each sex for 16 days, 13 weeks and 2 years. Results of these studies are summarised below.
44. In the 16-day studies, male rats (n=5) received furan at doses of 0, 5, 10, 20, 40 and 80mg/kg b.w. for a total of 12 days. Female rats and mice of both sexes received doses of 0, 10, 20, 40, 80 or 160 mg/kg b.w. Mottled and enlarged livers were observed at necropsy in male rats that received 20, 40, or 80 mg/kg furan, and in female rats given 40, 80 or 160 mg/kg. No lesions that were considered to be related to furan exposure were observed in mice.
45. For the 13-week studies, rats of each sex and female mice (n=10) received furan in doses of 0, 4, 8, 15, 30 or 60 mg/kg b.w. 5 days/week, while male mice received doses of 0, 2, 4, 8, 15 or 30 mg/kg b.w. Toxic lesions of the liver (bile duct hyperplasia, cholangiofibrosis, cytomegaly and degeneration of hepatocytes and nodular hyperplasia of hepatocytes) associated with furan exposure were seen in rats at all dose groups. The severity of these lesions increased with dose. In addition, kidney lesions (tubule dilatation and necrosis of tubule epithelium) were present in rats that received 30 or 60 mg/kg. Thymic atrophy and testicular or ovarian atrophy were also observed in rats exposed to 60 mg/kg furan. All groups of furan-treated mice had toxic liver lesions (cytomegaly, degeneration, and necrosis of hepatocytes), while mice receiving 30 or 60 mg/kg also had bile duct hyperplasia and cholangiofibrosis.

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46. In the 2-year study, rats of each sex (n=70) were administered 0, 2, 4 or 8 mg furan/kg b.w. 5 days/week for up to 104 weeks. Male and female mice (n=50) received 0, 8 or 15 mg/kg b.w. In rats, biliary tract fibrosis, hyperplasia and inflammation were seen at all doses tested, as well as hepatocellular cytomegaly, degeneration, hyperplasia, necrosis and vacuolisation. Mice treated with both doses of furan developed biliary tract fibrosis, hyperplasia and inflammation, and hepatocellular cytomegaly, degeneration and necrosis.
47. Wilson *et al.* (1992) investigated the effect of single or multiple doses of furan on hepatic toxicity and hepatocyte proliferation in F344 rats and B6C3F₁ mice (see annex 7). In the single dose study, male rats and mice (five per group) received corn oil or furan (30 mg/kg and 50mg/kg b.w., respectively) by gavage, and were sacrificed at various times between 12 hours and 8 days following administration. In the multiple dosing experiment, male and female animals (six per group) were gavaged daily 5 days per week for up to 6 weeks with either corn oil or doses of furan equivalent to the highest NTP bioassay dose (8 mg/kg b.w. for rats, 15 mg/kg b.w. for mice). The animals were sacrificed after 1, 3 or 6 weeks of treatment.
48. Following a single administration of furan, rats and mice developed necrosis that was evident 12 hours after treatment and peaked at 24 hours. By 48 hours, there was a sharp increase in hepatocyte proliferation in furan-treated animals. Livers had returned to near normal by 8 days. In the multiple-dosing studies, male and female rats, but not mice, had bile duct hyperplasia and metaplasia in areas of fibrosis along the subcapsular visceral surface of the left or caudate liver lobes following 6 weeks of treatment. Furan-treated mice and rats had higher hepatocyte labelling indices than control animals at weeks 1 and 6.
49. Isolated F344 rat hepatocytes in suspension were incubated with furan at concentrations calculated as similar to hepatic dosimetry *in vivo* (2-100 μ M) for 1-4 hours, and then placed in culture for 24 hours (Carfagna *et al.*, 1993). Furan resulted in cytolethality (5-70%), as evidenced by a significant increase in lactate dehydrogenase leakage and glutathione depletion in an exposure time- and concentration-dependent manner. This effect was prevented by adding the CYP inhibitor 1-phenylimidazole to the cultures, but was increased in hepatocytes from rats that had been pretreated with acetone to induce CYP2E1.
50. Incubation of isolated F344 rat hepatocytes with furan (2-100 μ M) for 1-4 hours produced a concentration-dependent decrease in ATP followed by cell death (Mugford *et al.*, 1997). This effect was prevented by the inclusion of 1-phenylimidazole in the suspensions. In the same study, rats were treated with furan (8,15 or 30 mg/kg po), sacrificed 24 hours later, and hepatic mitochondria were isolated and assessed for respiratory activity. Furan was seen to produce an uncoupling of hepatic mitochondrial oxidative phosphorylation that was prevented by pre-treating the rats with

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1-phenylimidazole. Similar results were observed in rat hepatocytes treated *in vitro* for 1-4 hours and then cultured for a further 24 hours. Further experiments indicated that the addition of oligomycin and fructose to cell suspensions also prevented furan-mediated ATP depletion and cytolethality. Fructose protects against chemically-induced mitochondrial lesions by increasing glycolytic ATP production, while oligomycin blocks ATPase activity to prevent hydrolysis of ATP generated by glycolysis.

51. Female B6C3F₁ mice were exposed to furan (4, 8 and 15 mg/kg b.w.) dissolved in corn oil by oral gavage 5 days/week for 3 weeks (Fransson-Steen *et al.*, 1997). Seven days prior to necropsy, mice were implanted with miniosmotic pumps that administered BrdU (30 µg/hour). Liver sections were immunohistochemically stained and labelling indices (LI) determined. Furan treatment resulted in a 2-3-fold increase in serum levels of alanine aminotransferase, sorbitol dehydrogenase and bile acids compared with control animals. These changes were accompanied by minor subcapsular inflammation and minimal histological evidence of necrosis at 8 and 15 mg/kg. Morphological examination of stained liver sections showed that the apoptotic index was significantly increased 6- and 15-fold above control values at 8 and 15 mg/kg, respectively. In addition, furan treatment led to a statistically significant dose-dependent increase in the hepatocyte LI. Compared with controls, mean values were 1.3-, 1.6- and 1.7-fold higher in mice treated with 4, 8 and 15 mg/kg furan, respectively. In a second experiment, mice were administered furan at 15 mg/kg and co-treated with aminobenzotriazole, a CYP inhibitor. These animals had a significantly lower LI and apoptotic index than animals treated with furan alone.

Carcinogenicity

Mouse

52. In an NTP bioassay (1993), B6C3F₁ mice of each sex (50 per group) were administered furan in corn oil by gavage (0, 8 and 15 mg/kg b.w.) 5 days per week for 104 weeks. In male mice, the incidence of hepatocellular adenomas and carcinomas, and benign pheochromocytoma was significantly increased at both doses. In females, a significant increase in hepatocellular adenomas was observed with both doses of furan, and a significantly increased incidence of hepatocellular carcinomas was seen at 15 mg/kg b.w.. The incidence of benign pheochromocytoma of the adrenal medulla was also increased at the top dose (see table 1).
53. Infant male B6C3F₁ mice received i.p. administration of furan in tricaprilyn during preweaning, either as a single dose of 400 mg/kg b.w. or six doses of 200 mg/kg b.w. (Johansson *et al.*, 1997; see annex 10). Animals were sacrificed at a range of time points between 28 and 95 weeks of dosing. The incidence of hepatic adenomas and carcinomas was 8/52 (15.4%) in the single-dose controls, 58/215 (27.0%) in the single-dose furan group,

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21/79 (26.6%) in multiple-dose controls, and 40/78 (51.3%) in the multiple-dose furan animals. Statistical analysis indicated that the incidence of hepatic neoplasia was significantly higher in mice given six doses of 200 mg/kg furan compared with vehicle-treated controls and animals given a single dose of 400 mg/kg furan. Apart from liver tumours, no other histopathological lesions were observed.

Rats

54. Male and female F344/N rats (70 per group) were administered furan dissolved in corn oil (0, 2, 4 or 8 mg/kg b.w.) by gavage 5 days/week for 102 weeks (NTP, 1993). Interim evaluations were performed on 10 rats from each group at 9 and 15 months. A high incidence of cholangiocarcinoma was seen in rats of both sexes with all doses of furan used.
55. In addition, the incidence of hepatocellular adenoma and carcinoma was significantly elevated at the two highest doses in male rats, while hepatocellular adenomas were significantly increased in females at the two highest doses. The incidence of mononuclear cell leukaemia was also elevated in male and female rats that received the two highest doses of furan (see table 2).
- 56. In a separate 2-year study (NTP, 1993), 50 male F344/N rats received 30 mg/kg furan in corn oil by gavage 5 days/week for 13 weeks. Groups of 10 animals were evaluated at the end of the furan administration period, and again at 9 months and 15 months. Cholangiocarcinomas were observed in all treated rats that survived for at least 9 months, while 2/10 had hepatocellular carcinomas at the 15-month evaluation. No details on statistics are provided with this study.**

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	Vehicle Control	8 mg/kg	15 mg/kg
Male			
2-year survival	33/50	17/50	16/50
Hepatocellular Adenoma or Carcinoma			
Overall rates	26/50	44/50	50/50
First incidence (days)	514	444	423
Logistic Regression tests *	P<0.001	P=0.001	P<0.001
Adrenal Medulla – Benign Pheochromocytoma			
Overall rates	1/49	6/50	10/50
First incidence (days)	723	701	423
Logistic Regression tests *	P=0.04	P=0.032	P=0.009
Female			
2-year survival	29/50	25/50	2/50
Hepatocellular Adenoma or Carcinoma			
Overall rates	7/50	34/50	50/50
First incidence (days)	646	446	360
Logistic Regression tests *	P<0.001	P<0.001	P<0.001

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Adrenal Medulla – Benign Pheochromocytoma				
Overall rates	2/50	1/50	6/50	
First incidence (days)	680	736	642	
Logistic Regression tests *	P=0.028	P=0.499	P=0.040	

Table 1. Incidences of neoplastic lesions in B6C3F₁ mice in the NTP 2-year bioassay.

P values associated with the trend test are beneath the control incidence. P values corresponding to pairwise comparisons between the controls and the dosed group are beneath the dosed group incidences.

	Vehicle Control	2 mg/kg	4 mg/kg	8 mg/kg
Male				
2-year survival	33/50	28/50	26/50	16/50
Hepatocellular Adenoma or Carcinoma				
Overall rates	1/50	5/50	22/50	35/50
First incidence (days)	660	729	613	490
Logistic Regression tests *	P<0.001	P=0.079	P<0.001	P<0.001
Cholangiocarcinoma				
Overall rates	0/50	43/50	48/50	49/50
First incidence (days)	-	470	254	421
Logistic Regression tests *	P<0.001	P<0.001	P<0.001	P<0.001
Mononuclear Cell Leukaemia				
Overall rates	8/50	11/50	17/50	25/50
First incidence (days)	645	384	520	421
Logistic Regression tests *	P<0.001	P=0.267	P=0.027	P<0.001
Female				
2-year survival	34/50	32/50	28/50	19/50

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Hepatocellular Adenoma or Carcinoma					
Overall rates		0/50	2/50	4/50	8/50
First incidence (days)		-	697	688	644
Logistic tests *	Regression	P<0.001	P=0.224	P=0.048	P<0.001
Cholangiocarcinoma					
Overall rates		0/50	49/50	50/50	48/50
First incidence (days)		-	436	388	143
Logistic tests *	Regression	P<0.001	P<0.001	P<0.001	P<0.001
Mononuclear Cell Leukaemia					
Overall rates		8/50	9/50	17/50	21/50
First incidence (days)		556	602	557	238
Logistic tests *	Regression	P<0.001	P=0.526	P=0.034	P=0.008

Table 2. Incidences of neoplastic lesions in F344/N rats in the NTP 2-year bioassay.

* P values associated with the trend test are beneath the control incidence. P values corresponding to pairwise comparisons between the controls and the dosed group are beneath the dosed group incidences.

57. Groups of 10 or 12 adult male F344 rats were administered furan (30 mg/kg b.w.) by gavage five days/week for 6, 9, 12 and 13 weeks and monitored for up to 16 months (Elmore and Sirica, 1993). In total, 9/10 animals treated for 13 weeks had hepatic adenocarcinomas in the right/caudate lobe of the liver, while 3/10 had adenocarcinomas in the median/left lobe. Hepatocellular carcinomas were only observed in rats treated with furan for 13 weeks (2/10), and one primary hepatic cholangiocarcinoma was also detected. Of note, 26 of the 27 furan-induced hepatic adenocarcinomas examined showed evidence of small intestinal differentiation, as indicated by the presence of goblet cells, Paneth cells and serotonin-positive neuroendocrine cells.

58. Administration of furan by gavage to male F344 rats at doses of 30-45 mg/kg b.w. 5 days/week for 2-6 weeks resulted in the development of metaplastic small intestinal-like glands closely resembling the crypts of Lieberkühn of normal rat small intestine (reviewed by Sirica, 1996). These glands were derived from putative hyperplastic bile ductile-like progenitor structures, and preferentially formed in the right and caudate liver lobes. Administration of furan at 30 mg/kg b.w. 5 times/day for 9-19 weeks resulted in the preferential development of primary hepatic adenocarcinomas, which formed in the right/caudate liver lobes at

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incidences ranging from 70-100%. These adenocarcinomas were characterised by small intestine mucosal cell differentiation.

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ANNEX 2 TO CC/05/22

DRAFT MINUTES OF COM MEETING ON 13 OCTOBER

ITEM 5 MUTAGENICITY OF FURAN (MUT/05/20)

18. No interests were declared.

19. The COM had been asked for advice on the mutagenicity of furan by the COT who were considering research that would be helpful regarding the risk assessment of this contaminant. Members agreed there were no clear structural alerts but metabolism mediated by CYP2E1 could lead to epoxide formation which was consistent the finding of cis-2-butene-1,4-dial. It was noted that carbon dioxide was a major metabolite which was indicative of furan ring opening occurred. However there were no quantitative data on metabolism available.

20. The Committee considered that the available mutagenicity tests of furan in bacteria had been adequately conducted. It was noted that CYP2E1 would not be significantly induced in Arochlor 1254- induce rat liver S-9 mix. The positive result in Salmonella typhimurium TA 104 with cis-2-butene-1,4-dial was consistent with this compound being a reactive aldehyde. Members noted the positive results reported in chromosomal aberrations tests in CHO cells in both the presence and absence of exogenous metabolic activation but commented that the concentrations where positive results had been identified (ca 125 mM and 250 mM) were considerably greater than the OECD upper limit of 10 mM for non cytotoxic test materials. There was no information on cytotoxicity in the NTP test report on cytotoxicity.

21. Members considered that the L5178Y mouse lymphoma assay reported by the NTP had been conducted to standards acceptable at the time using plating in soft agar but had not undertaken a trial in the presence of an exogenous metabolising fraction. It was considered the cells might have expressed some CYP2E1 activity. A negative response had been reported in trial 1 but a dose related increase in mutation frequency was seen in trials 2 and 3 which included dose levels where there was only a small decrease in relative total growth. It was noted that dose levels used in trials 2 and 3 were very high at around 250-500 mM. Members agreed that in view of the positive results at non-cytotoxic doses that this study should be considered as positive and that furan should be regarded as having in-vitro mutagenic activity.

22. The Committee considered the available in-vivo mutagenicity studies. The key in-vivo bone marrow chromosomal aberration assay was undertaken as part of the NTP programme on furan. The investigators had used several dose levels and multiple sampling times. The two trials which had used an extended harvest time of 36 hours reported a statistically significant increase of in chromosome aberrations (12 and 11 fold respectively) at 250 mg/kg bw

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administered by gavage. No information on toxicity was available. However it was noted that other published data suggested that a single oral gavage dose of furan at 50 mg/kg bw resulted in moderate histopathological signs of liver toxicity whilst a dose level of 250 mg/kg bw resulted in extensive hepatocellular necrosis and was at the limit of lethality. Members agreed that the data from this study could not be interpreted with certainty because of the confounding effects of toxicity at the dose level used.

23. The Committee agreed that negative results had been obtained in an in-vivo liver UDS studies in rats and mice. Covalent binding studies using oral or intraperitoneal doses of radiolabelled 2,5-¹⁴C-furan in rats was available. There was evidence for dose related binding of furan to protein. Members considered the studies were limited by use of low specific activity radiolabelled furan, low sensitivity of radiolabel analysis and the incorporation of carbon dioxide formed from furan into intermediary metabolism. The results of in-vitro studies of the reaction of cis-2-butene-1,4-dial with deoxyguanosines indicated reactions with adenosine, guanine and cytosine but not thymidine. The authors had proposed that cis-2-butene-1,4-dial might form DNA cross links.

24. Members agreed that no definite conclusions could be drawn from the studies of proto-oncogene mutation spectra in hepatocellular adenomas and carcinomas from control and furan treated mice.

25. The Committee concluded that furan should be regarded as an in-vitro mutagen but there was insufficient evidence to reach a conclusion on the available in-vivo mutagenicity data.

26. The Chairman noted that the Food Standards agency had asked for advice on what research could be used to support a threshold approach to risk assessment. Members felt that adequate in-vivo mutagenicity testing would be useful. It was suggested this might include bone marrow micronucleus test or DNA binding studies in cancer target organ tissues. It was noted that the critical carcinogenic effect was cholangiocarcinoma and there would be difficulties in isolating bile duct cells during DNA binding studies. There was some discussion regarding whether a study using Accelerator Mass Spectrometry (AMS) would be appropriate. This would require the formation of stable DNA adducts. The potential for DNA-protein cross links would need to be considered. Members considered that further consideration of the mechanisms of in-vitro mutagenicity and the possible use of scavaging agents (e.g N-acetylcysteine) to investigate whether the primary mode of action was cytotoxicity would also be useful. It was noted that any further in-vitro studies would need to consider the selection of solvents carefully as many commonly used solvents (e.g DMAO, acetone, ethanol) would compete with furan for metabolism via CYP2E1.

27. It was agreed that the COM advice would be passed to COC for consideration during its discussions on furan. The COM and COC advice

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would be reported back to COT as conclusions for inclusion in a COT statement.

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ANNEX 3 TO CC/05/22

Butterworth B E, Sprankle C S, Goldsworthy S M, Wilson D M & Goldsworthy T L; Expression of *myc*, *fos* and *Ha-Ras* in the livers of furan-treated F344 rats and B6C3F₁ mice. *Molecular Carcinogenesis* 9:24-32 (1994)

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ANNEX 4 TO CC/05/22

Sirica A E, Radaeva S & Caran N; NEU overexpression in the furan rat model of cholangiocarcinogenesis compared with biliary ductal cell hyperplasia. Am J Pathol 151:1685-1694 (1997)

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Lai G-H, Radaeva S, Nakamura TA & Sirica A E; Unique epithelial cell production of hepatocyte growth factor/scatter factor by putative precancerous intestinal metaplasias and associated “intestinal-type” biliary cancer chemically induced in rat liver. Hepatology 31:1257-1265 (2000)b

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ANNEX 5 TO CC/05/22

Moser G J, Foley J, Burnett M, Goldsworthy T L & Maronpot R; Furan-induced dose response relationships for liver cytotoxicity, cell proliferation and tumorigenicity.

The paper in this Annex has been submitted for publication following peer review and will not be made available prior to publication.