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**Executive Summary M1 –M36**

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<td>Project coordinator name</td>
<td>Prof. Dr. Wolfgang Dekant</td>
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<td>Project coordinator institution name</td>
<td>University of Würzburg, Germany</td>
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Executive summary

In a recent investigation, the U.S. Food and Drug Administration (FDA) identified the chemical furan in a variety of food items that undergo heat treatment. Furan is a potent hepatotoxicant and liver carcinogen in rodents.

Although data on human intake of furan is limited, it appears that there is a relatively narrow margin between human exposure and doses which cause liver tumors in rodents, suggesting that the presence of furan in food may present a potential risk to human health. However, the presently available data on furan toxicity is insufficient to perform a risk assessment and more research regarding the mechanism of furan carcinogenicity is needed.

The project addressed modes of action for tumor induction in liver by the food contaminant furan, which is formed by processing of food resulting in widespread human exposure. There is uncertainty regarding the relevance of tumors induced in rodents to human risk assessment because the mechanisms are unclear. The research addressed the role of DNA and protein binding of furan, oxidative DNA damage, non-genotoxic alteration of proliferation and apoptosis, cyogenetics and cytotoxicity in furan-mediated liver toxicity and carcinogenicity. A combination of in vivo and in vitro systems, analytical chemistry, cell biology and "omics" technologies was applied. In rodents in vivo, extent and dose-dependence of covalent binding to DNA, cyogenetic changes and geno- and cytotoxicity in target cells in the liver were addressed after oral administration of furan. In addition, the induction of oxidative DNA-modifications and mechanisms of mutations were investigated in genetically modified rodent models. These in vivo studies characterize the mode of action of furan and also address irreversible metaplasia, changes in cell signaling and inflammation. The interaction of these effects with possible genetic changes in liver cells including aspects of forced cell proliferation were included. The in vivo work is complemented by studying mode of mutation of furan and its metabolite cis-2-butene-1,4-dial in cell culture models resembling the target cells. The content of furan in food was determined and human exposures was assessed using probabilistic modeling. Mechanisms of formation of furan in food may open ways to reduce exposures. The results provided data on the mode-of-action of furan induced liver carcinogenesis as a basis for a conclusive assessment of health risks in humans due to dietary exposure. The combination of these findings provide a risk/benefit analysis and a scientific basis to justify limits for human furan exposures.
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Objectives

Since the mechanisms of carcinogenic activity of furan in rodents are not well understood, the objectives of this project are to generate relevant mechanistic information as a support for the ongoing risk assessment of human furan exposures with food. The importance of mode-of-action research on furan is underscored by the comparatively small difference in the estimated human exposures and the doses of furan, which cause carcinoma in the liver of experimental animals.

A detailed elucidation of genotoxic and non-genotoxic mechanisms and their possible dose-response relationships and interconnectivity are of fundamental importance for a reliable risk assessment.

The final outcome of the project will be a careful assessment of the genotoxic potential of furan in the target cells in vivo for carcinogenicity, generation of information on the potential relevance of DNA lesions and the tissue environment changes that may exacerbate mutations. The methods applied includes state-of-the-art analytical procedures to quantify furan induced DNA-adducts in very low concentrations and other DNA-damage or cytogenetic changes, biomarkers, gene-arrays and modern cell biology to characterize furan induced changes in cellular function in the target cells of carcinogenesis in relevant species.
Following major objectives are addressed in this project:

1. Characterization and quantitation of DNA- and protein binding of furan in liver of rats and mice over a wide dose range. Both responses after single and after repeated exposures were assessed. (WPs 1.1 and 1.2)

2. Analysis of biomarkers of toxicity, genotoxicity and epigenetic changes affecting gene expression, tissue structure changes and cell proliferation in target cell populations of furan in order to elucidate cell-specific mechanisms. Again, special attention will be given to determine the dose dependence of the effects. (WPs 2.1. and 2.2)

3. Assessment of the genotoxic and clastogenic potential of furan in rodent liver by comet assay, cytogenetics and biomarkers of genetic damage. (WPs 3.1 and 3.2)

4. Assessment of the role of the accumulation of oxidative DNA damage in the mechanism of neoplastic transformation by means of a genetically modified mouse models. (WP 4)

5. Detailed analysis of gene expression changes. (WP5)

6. Characterization and quantitative assessment of genetic changes induced by furan and cis-2-butene-1,4-dial in mammalian cells with special consideration on mechanisms for induction of gene mutations and cytogenetic changes. (WPs 6.1, 6.2 and 6.3)

7. Characterization of furan and cis-2-butene-1,4-dial induced effects on toxicity parameters, and DNA-damage in cell culture models for the specific target cells of furan in order to elucidate cell-specific mechanisms. (WP 7)

8. Analysis of furan in food as a better basis for exposure assessment from food and mechanisms of formation during food processing. (WPs 8.1 and 8.2)

9. Provision of a risk assessment of furan in food. (WP 9)

Fig. 1: Graphical presentation of the components showing their interdependencies
Work performed

In the first year, research activities were devoted to performing a 28-day repeated dose study with oral dosing of furan as a central repository for tissues to be analysed. In addition, study conditions regarding both in vivo and in vitro genotoxicity studies were optimized and preliminary results were generated. A database on levels of furan reported in food was assembled and mechanistic studies to investigate mechanisms of formation of furan in food were performed using stable isotope labeled precursors.

A Web-site (http://www.furan-ra.toxi.uni-wuerzburg.de/), which is regularly updated with results (deliverables and presentations presented by partners at the meetings) has been created. The web-site contains a public area and a password protected area for project participants, EC-representatives and external advisors.

In the second reporting period following work was performed:

To assess DNA-binding of furan, an in vivo study was conducted in which male Fischer rats were treated with $^{14}$C-furan at a known carcinogenic dose and at a dose close to human exposure. DNA was extracted from livers of furan treated animals and analysed by accelerator mass spectrometry.

Subacute liver toxicity of furan in rats was characterized regarding changes in histopathology, clinical chemistry, metabonomics, genotoxicity, as well as non-genotoxic alterations in DNA-methylation, gene expression and cell proliferation.

The genotoxic activity of furan in B56C3F1 mice was investigated. Toxicity and genotoxicity end-points were evaluated in spleen and liver of animals receiving furan by the oral route along a four weeks period. Genotoxicity end-points were also assessed in animals receiving furan as an acute high dose.

In addition parallel measurements of DNA oxoG were performed in several organs of these Furan-treated mice. To investigate whether furan exposure increased oxidative DNA damage, C57BL/6 mice defective in the base excision repair Ogg1 gene were also used. Experiments were performed in Ogg1$^{-/-}$ and wild-type mice using the same protocol of furan exposure. DNA 8-oxoG levels were measured in several organs and possible correlations with Furan-induced liver toxicity and genotoxicity were analysed.

Mutagenesis induced by furan and its metabolite cis-2-butene-1,4-dial were investigated in mammalian cells in culture at two different gene loci (i.e. hprt and tk). In parallel, DNA single strand breaks (ssb) and 8-oxoG levels were measured. The expression of a set of DNA repair genes was also monitored.

Two immediate precursors in the formation of furan during heat treatment of food were synthesized and their conversion into furan was investigated Strategies such as use of antioxidants or inert atmosphere were investigated to suppress lipid oxidation and hence reduce furan formation in food.

β-carotene and retinol were investigated as new highly potential precursors of furan. Enhancement of furan formation from unsaturated lipids in the presence of several prooxidants such as ascorbic acid, b-carotene, and transition metals were evaluated. Kinetics of furan formation was studied in food systems (pumpkin puree, carrot and orange juice) Activation energies were determined from obtained kinetic data.

Over one hundred food samples from the categories carrot and prune juices, nutrition drinks and bakery products were collected and analysed on furan levels. Attempts have been made to correlate the furan levels found with the ingredients and the production processes.

In the third reporting period following work was performed:

P1: Work performed in the third reporting period involved characterization of radioactive compounds to distinguish between metabolic incorporation and covalent binding and the design of a scheme of metabolic pathways leading to biliary metabolites of furan.
P2: The immunofluorescent detection of foci of phosphorylated histone H2AX (γ-H2AX), together with a radiation-modified comet protocol, were applied to investigate the formation of DNA-DNA crosslinks in mouse liver and spleen cells following acute and subacute furan administration.

A transcriptomic analysis on selected toxicity and DNA damage response genes in mouse liver was performed after 4 weeks exposure to a carcinogenic furan dose.

The organ specificity of oxidative DNA damage associated with Furan exposure was investigated in a set of experiments performed in C57BL/6 mice. In addition the possible relationship between 8-oxoG accumulation in the liver and the inflammatory response in this organ was explored by immunohistochemistry. Finally Furan-induced chromosomal damage in splenocytes was demonstrated to be independent from induction of oxidative DNA damage to the spleen.

Final data were obtained on the mutagenic potential of furan and its metabolite cis-2-butene-1,4-dial in two mammalian cell lines at two different loci. Only cis-2-butene-1,4-dial induced a significant increase in mutation frequency at both loci. To gain mechanistic insights into the mutagenic activity of cis-2-butene-1,4-dial the TK mutation assay was exploited by enumerating the small and large mutant colonies. In addition, in the same cell system the induction of micronuclei was investigated in the same mutagenic dose range.

P3: The effects of 2-butene-1,4-dial (BDA), the major metabolite of furan, on primary rat hepatocytes and the rat hepatoma cell line H4IIE were tested.

P4: Changes in the expression of genes involved in DNA damage response were investigated following furan exposure including the dose response. Gene-specific methylation patterns and global DNA methylation were determined following furan treatment using furan induced cholangiocarcinoma samples as a reference.

P5: Completion of cytogenetic profile of furan and its key metabolite cis-2-butene-1,4-dial in vitro in human lymphocytes and two human lymphoblastoid cell lines from group “A” Fanconi’s anemia patients [LFA145(-/-); LFA8(+/−)] and two cell lines from normal individuals [LFA194(+/+); LFA195(+/+)] which served as controls. The use of Fanconi’s anemia cells has been justified by their hypersensitivity to DNA cross-link agents since cis-2-butene-1,4-dial is a DNA cross-linking agent. Furthermore, a panel of DNA-repair-defective Chinese hamster cells which result in a greatly enhanced cytogenetic effects of the compound assayed when a relevant key enzyme in a specific DNA repair pathway is missing or compromised was also employed, e.g. (UV4) defective for nucleotide excision; (EM9) defective for base excision repair; (irs1SF) defective for homologous recombination; (V3) defective for non homologous end-joining recombination (NHEJR).

In vivo, following oral administration of furan in rats for 28 days, evaluation of chromosome aberrations and micronuclei in bone marrow erythrocytes and chromosomal aberrations in resting G0 splenocytes was performed. Furthermore, DNA damaging activity under the above reported experimental conditions has been evaluated by the alkaline comet assay in blood, bone-marrow and liver cells.

P6: About one hundred samples were collected (fruit and vegetables juices, nutrition drinks and bakery products) and analysed for furan content. The results were combined with those from the preceding years and added to the existing data base on furan levels in food (project deliverable D8.1) to create the project deliverable D8.2. The analytical method was validated and the results were described in a validation report (project deliverable D8.3).

Results achieved so far

In the first year, the 28-day oral toxicity study only showed minor liver damage induced by furan both in rats and in mice, but analysis of the tissues and blood samples already indicated changes in gene expression and bile acid excretion. In addition, a number of biliary
metabolites formed from furan or its reactive metabolite were characterized. Samples for analysis by accelerator mass spectrometry have been generated and were analysed in spring 2008. The genotoxicity studies performed showed equivocal results with furan itself but a clear response with the reactive furan metabolite when added directly to the cell culture media. The summary of furan concentrations shows a wide range of encountered furan concentrations in food and identified some further needs to analyse specific food items for furan content. The mechanistic studies have indicated specific pathways of furan formation which require confirmation in further studies.

The performed studies are in line with the workplan of the project and will serve as a basis for further development of mode-of-action-models for furan. The results will provide data on the mode-of-action of furan induced liver carcinogenesis as a basis for a conclusive assessment of health risks in humans due to dietary exposure. Combining these findings will provide a risk/benefit analysis and a scientific basis to justify limits for human furan exposures. Results of the project were published in peer-reviewed scientific journals to make the scientific and regulatory community aware of the project results and as a measure of project success. Assessment and evaluation of WP results and progress towards the objectives were monitored by the participants in each workpackage.

In the second year following results were obtained:

The $^{14}$C content in DNA extracted from low and high dose animals was significantly increased in a dose dependent manner. While these results indicated that furan may bind to DNA, it was important to exclude the possibility that the increased $^{14}$C content in DNA extracted from furan dosed animals was due to metabolic incorporation into DNA. Detailed analysis indicated that radioactivity in DNA did not coelute with normal nucleoside suggesting covalent modification.

No overt signs of liver toxicity were evident by histopathology, clinical chemistry and metabonomics after oral administration of furan for 28 days. However, a dose-dependent increase in cell proliferation was detected specifically in areas located near the edge of the liver lobes, with the caudate lobe being particularly susceptible. Consistent with these changes, treatment with furan led to up-regulation of a number of apoptosis and cell cycle related genes. No global DNA methylation change or gene-specific methylation change were found at the dose levels employed.

In vivo genotoxicity assays in B6C3F1 mice provided evidence of genotoxic effects after repeated oral exposure with furan. In particular, a dose related, statistically significant increase of micronuclei was observed, following in vitro stimulation, in binucleated splenocytes from animals exposed in vivo (4 to 15 mg/kg bw). No similar increase was seen in splenocytes from animals administered with single high furan doses (15 to 250 mg/kg bw) Conversely, in liver a significant induction of DNA damage, as detected by comet assay, was only observed after high dose (250 mg/kg b.w.) acute exposure. Furan treatment was associated with a marginal increase in oxidative DNA damage, limited to some organs (mainly the lung). When oxidative DNA damage associated with furan exposure was investigated in animals defective in the repair of 8-oxoG (the Ogg1$^{-/-}$ C57Bl/6 mice) no amplification of the levels of this oxidized purine was observed.

A dose-related increase in hprt mutation frequency was observed after treatment of V79 cells with cis-2-butene-1,4-dial, but not with furan. Furan induced a dose-related increase in tk mutation frequency in L5178Y cells, but at high cytotoxic doses. Furan and its metabolite did not induce directly DNA ssb but produced dose-related increases in 8-oxoG. Up-regulation of 3-methyladenine DNA glycosylase, a marker of inflammation, was detected after exposure to furan.

Findings on in vitro cytogenetic analysis of furan and its metabolite in “normal” and Fanconi’s anemia cell lines support for a genotoxic (clastogenic) activity of furan following metabolic conversion through its key metabolite, cis-2-butene-1,4-dial.
Analyses of furan levels in fruit juices confirm the information from the literature (i.e., a wide variation), whereas levels in nutrition drinks are much lower than found in the literature. Levels in bakery products are higher than reported in the literature. There seems to be a correlation between the heat treatment of the carrot juice samples and the furan levels. No correlation between the nutrition drink ingredients and the furan levels could be found, though. Studies performed up to now have brought already a good understanding of the mechanisms of furan formation during heat treatment of food which allows suggesting of several mitigation strategies.

In the third year following results were obtained:

P1: The results obtained demonstrate that only a minor amount of radiocarbon is associated with normal nucleosides, suggesting that the increase in $^{14}$C-content in DNA extracted from livers of rats treated with $^{14}$C-furan is due to covalent binding.

In summary, chemical characterization of furan metabolites in bile provided further evidence to suggest that GSH-conjugates and degraded protein adducts are major in vivo metabolites of furan, consistent with the hypothesis that cytotoxicity mediated through binding of cis-2-butene-1,4-dial to critical target proteins is likely to play a key role in furan toxicity and carcinogenicity. Although no toxicity data are available so far, the metabolites identified in bile are assumed to be less toxic than cis-2-butene-1,4-dial and the initial mono-GSH conjugate. Biliary toxicity of furan may thus not to be a result of exposure of the biliary epithelium to high concentrations of reactive metabolites, but may occur secondary to hepatocyte damage.

P2: In studies in B6C3F1 mice, repeated oral furan administration was shown to induce in vivo primary DNA damage in spleen cells, which evolved in double strand breaks following mitogen stimulation of cells in vitro. Direct evidence of induction of DNA damage in the liver was only obtained following a high dose, acute exposure; upregulation of DNA damage response genes was however observed after repeated oral administration of a lower, non toxic furan dose.

Organs in which oxidative DNA damage accumulates following a 28-day Furan exposure were identified. While no treatment related increases in DNA 8-oxoG were detected in the brain, kidney, small intestine or spleen of wild-type C57BL/6 mice exposed to any Furan dose, 1.9- and 1.7-fold increases in 8-oxoG levels were observed in the lung and in the liver, respectively. Low doses of Furan exposure (8 and 15 mg/kg) were found to be associated with increased proliferation of hepatocytes and Kupffer cells. The highest Furan dose (30mg/kg) led to portal, periportal and intralobular damage, as shown by fatty degeneration, presence of apoptotic cells as well as necrotic hepatocytes. In addition inflammatory cells were found to surround enlarged and fibrotic portal tracts. Our data indicate that increased levels of 8-oxoG are mostly associated with increased cell proliferation in the liver. Furan-induced clastogenic activity was confirmed to be independent from the ability to induce oxidative DNA damage.

The experiments to investigate mutagenesis of Furan and its metabolite in V79 and L5178Y rodent cells at the hprt and TK locus respectively were completed. A significant increase in mutation frequency was only detected in the case of cis-2-butene-1,4-dial in a very narrow range of concentrations in both mutation assays. In the case of the TK assay the enumeration of small and large colonies showed an increase in the relative number of small colonies indicating the occurrence of large deletions/gross rearrangements. This mode of action was also confirmed by the significant increase in micronuclei frequency in the same range of doses where the mutagenic activity was displayed. DNA 8-oxoguanine levels increased significantly upon treatment with either Furan or cis-2-butene-1,4-dial suggesting that DNA oxidation thus not play a major role in the mutagenic activity of cis-2-butene-1,4-dial.
P3: The effects of 2-butene-1,4-dial (BDA), the major metabolite of furan, on primary rat hepatocytes and the rat hepatoma cell line H4IIE were tested. With an EC50 of 0.59 mM a three-fold higher sensitivity in H4IIE was determined.

The spontaneous reaction of BDA with glutathione resulted in a multitude of products. As this reaction mixture showed cytotoxic effects on HepG2, the major metabolites were isolated and tested separately for cytotoxicity.

P4: Modulation of DNA damage-related genes and limited DNA-methylation change were found at a relatively high dose (30 mg/kg bw) but not at the lower doses (up to 2 mg/kg bw).

P5: In vivo, following oral administration of furan in rats for 28 days positive results obtained for induction of chromosomal aberrations and for DNA breakage ( Comet assay) were observed in G0 splenocytes isolated from spleen of control and furan-treated animals, and stimulated to grow in vitro following stimulation with concanavalin A® and in liver (high dose, 4 weeks treatment + 1 weeks recovery) respectively. Negative results were obtained for induction of micronuclei, SCE’s and chromosomal aberrations in bone-marrow cells and for DNA breakage in bone marrow and blood cells. Negative findings obtained for these end-points in the proliferating bone marrow cells could be ascribed to a limited availability of furan metabolites in this tissue at dose-levels employed and a selective elimination of cells bearing aberrations due to proliferation. Conversely, positive findings observed in splenocytes which are resting in the G0 phase of cell cycle and therefore exposed to the test agent or its metabolites for the whole length of treatment without any selection of damaged cells as in the case of proliferating cells (e.g. bone-marrow cells) allows accumulation of DNA lesions which are then converted into cytogenetic damage, following DNA repair activities. On the bases of the results obtained furan proved to be an in vivo genotoxin in rats under the reported experimental conditions, though in “non-conventional” genotoxicity assays.

In vitro, furan did not induce chromosomal aberrations in “normal” human lymphocytes in the presence of S9 metabolism, though treatments were performed at the maximum allowable conditions in vitro (e.g. 3 hours treatment and 10 mM highest dose-level). Conversely, furan induced elevated levels of chromosomal aberrations in Fanconi’s anemia cell lines at 10 mM concentration in the presence of S9 metabolism (CYP2E1). Due to the hypersensitivity of Fanconi’s anemia cell lines to DNA cross-linking agents, clastogenicity of furan is likely to be caused by its DNA cross-linking key metabolite cis-2-butene-1,4-dial (BDA). Negative results obtained in in human lymphocytes in the presence of S9 metabolism (CYP2E1) following treatment with Fur can be explained by the fact that furan is converted into DNA-reactive metabolites but at a rate not sufficient to be detected by “conventional” genotoxicity assay. Alternatively, Fanconi’s Anemia cells which are defective in the pathway to repair DNA cross-links are sensitive enough to detect furan genotoxic metabolites. In the case of cis-2-butene-1,4-dial it was hardly detected as clastogenic compound in “normal” human lymphoblastoid cells only in the long treatment (24 hours) at the highest dose-level selected for scoring (23.2 μM). When using Fanconi’s anemia cell lines which are hypersensitive to DNA cross-linking agents, cis-2-butene-1,4-dial proved to be highly clastogenic even in the short treatment time (4 hours), thus indicating that cis-2-butene-1,4-dial is likely acting as a DNA cross-linking agent. This observation is further supported by results obtained when cell cultures are allowed to repair for 12 hours following 24 hour treatment (36 hr sampling time). The incidence of chromosomal aberrations return to control values in the “normal” lymphoblastoid cell line LFA195 (+/+) while in Fanconi’s anemia cells elevated levels of chromosomal damage still remains.

Results obtained indicate that furan is a subtle in vitro promutagen whose genotoxicity is likely to be caused by its DNA cross-linking key metabolite cis-2-butene-1,4-dial. It can be detected as in vitro genotoxin in “non conventional” cytogenetic assays due to the limited quantities of active metabolite converted in vitro and its elevated reactivity with key structures of cells (e.g. protein, DNA etc.). Furthermore, due the elevated toxicity of cis-2-butene-1,4-dial it can be detected as genotoxin only in a very narrow range of dose-levels (15 – 50 μM).
This last property probably explains the numerous negative findings reported in literature.

P6: Analysis of furan in fruit and vegetables juices showed that elevated furan levels were found in carrot juices with relatively high pH-values and prune juices. Other flavours of the brands that have high furan levels in their carrot or prune juices had low furan levels. Carotene in combination with sterilisation is a possible source of furan in carrot juices. The prune drying process is a possible source of furan in prune juices. Babies may have high exposure to furan when their carrot juice consumption is high (0.25 litre per day). Adults are not at risk when consuming carrot or prune juices.

Only one type of pharmaceutical nutrition drinks showed high furan levels, regardless of the flavour. Other types of the same brand and all types of other brands had low furan levels. Patients that exclusively consume the high furan level type at the recommended intake (substitution of all meals by nutrition drinks) have a high exposure to furan. None of the nutrition drinks that are available in the cooled shelves of supermarkets had detectable furan levels. No furan source could be detected in the pharmaceutical nutrition drink. Conservation heat treatment may play a role in furan formation. Heat treatment is applied in pharmaceutical nutrition drinks, not in the short shelf life supermarket products.

Furan determination in bakery products turned out to be irreproducible, possibly because of inhomogeneous furan distribution in the products. The highest furan levels were found in wholegrain products. The baking process is the probable furan cause. Consumers of even the highest furan containing products have no significant furan exposure, though.

The furan method performance was evaluated through a validation process. The method performs well for liquid and viscous products. For solid products, however, the reproducibility is very low, leading to elevated limits of detection and quantification.

Summary of project results
Due to genotoxicity and DNA-binding of furan established in the project, the current risk assessment for furan requires application of a linear dose response relationship. Exposures to furan in adults are estimated to be less than 0.5 μg/kg bw/day, data from this project also support a similar intake of furan in children. Due to the high incidence of tumors induced by furan in rats even at the lowest applied dose, a linear extrapolation of the animal data to calculate possible human tumor risks is uncertain. When using a margin-of-exposure assessment, MoEs to a dose inducing a significant tumor incidence are calculated as only 1000 to 2000. The low MoEs warrant further reductions in furan-exposures.