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Appendix 2 - Deliverables

FURAN-RA

Publishable 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 proposal is addressing 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 is addressing the role of DNA and protein binding of furan, oxidative DNA damage, non-genotoxic alteration of proliferation and apoptosis, cytogenetics 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 will be applied. In rodents in vivo, extent and dose-dependence of covalent binding to DNA, cytogenetic changes and geno- and cytotoxicity in target cells in the liver are addressed after oral administration of furan. In addition, the induction of oxidative DNA-modifications and mechanisms of mutations are investigated in genetically modified rodent models. These in vivo studies will 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 will be 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 will be determined and human exposures will be assessed using probabilistic modeling. Mechanisms of formation of furan in food may open ways to reduce exposures. 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.
Appendix 2 - Deliverables

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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 will be 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)
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 B6C3F1 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, β-carotene, and transition metals was 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.

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 will be 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. All results of the project will be 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 will be 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 indicate that furan may bind to DNA, it is important to exclude the possibility that the increased $^{14}$C content in DNA extracted from furan dosed animals is 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.