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Transcriptome analysis provides new insights into liver changes induced in the rat upon dietary administration of the food additives butylated hydroxytoluene, curcumin, propyl gallate and thiabendazole

Rob Stierum [a,*], Ana Conesa [a,1], Wilbert Heijne [a,2], Ben van Ommen [a], Karin Junker [b], Mary P. Scott [c,d], Roger J. Price [c,d], Clive Meredith [c,3], Brian G. Lake [c,d], John Groten [a,4]

[a] TNO Quality of Life, Business Unit Biosciences, P.O. Box 360, 3700 AJ Zeist, The Netherlands
[b] TNO Quality of Life, Business Unit Quality and Safety, Zeist, The Netherlands
[c] BIBRA International Ltd., Woodmansterne Road, Carshalton, Surrey, SM5 4DS, UK
[d] Centre for Toxicology, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, England

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ABSTRACT

Transcriptomics was performed to gain insight into mechanisms of food additives butylated hydroxytoluene (BHT), curcumin (CC), propyl gallate (PG), and thiabendazole (TB), additives for which interactions in the liver can not be excluded. Additives were administered in diets for 28 days to Sprague–Dawley rats and cDNA microarray experiments were performed on hepatic RNA. BHT induced changes in the expression of 10 genes, including phase I (CYP2B1/2; CYP3A9; CYP2C6) and phase II metabolism (GST μ2). The CYP2B1/2 and GST expression findings were confirmed by real time RT-PCR, western blotting, and increased GST activity towards DCNB. CC altered the expression of 12 genes. Three out of these were related to peroxisomes (phytanoyl-CoA dioxygenase, enoyl-CoA hydratase; CYP4A3). Increased cyanide insensitive palmitoyl-CoA oxidation was observed, suggesting that CC is a weak peroxisome proliferator. TB changed the expression of 12 genes, including CYP1A2. In line, CYP1A2 protein expression was increased. The expression level of five genes, associated with p53 was found to change upon TB treatment, including p53 itself, GADD45α, DN-7, protein kinase C β and serum albumin. These array experiments led to the novel finding that TB is capable of inducing p53 at the protein level, at least at the highest dose levels employed above the current NOAEL. The expression of eight genes changed upon PG administration. This study shows the value of gene expression profiling in food toxicology in terms of generating novel hypotheses on the mechanisms of action of food additives in relation to pathology.

1. Introduction

In the European Union, food additives are authorised for use provided that they do not impose any hazard upon consumers at doses used. Intake levels of individual food additives are considered to be safe at the Acceptable Daily Intake (ADI). The ADI is derived from the No Observed Adverse Effect Levels (NOAEL) established in laboratory animals taking into account a safety factor for intra and interspecies differences. Possible joint actions or interactions are not taken into account in this uncertainty factor. Recently, the International Life Sciences Institute (ILSI)-Europe Acceptable Daily Intake Task Force undertook an evaluation of the potential interactions occurring between 350 currently approved food additives or preservatives (Groten et al., 2000). At first, a rationale, based on the criteria used by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) to establish group ADI values was adopted to select 65 additives for further consideration. Thereafter, the possible joint actions or interactions of these 65 additives were assessed based on toxicodynamic and toxicokinetic considerations. The following criteria were considered to determine those additives and effects which should be subject to detailed consideration: the additive was approved in the EU and had been allocated an ADI number; the additive showed clear organ toxicity in liver or kidney at doses above the NOAEL; a joint action or interaction was considered possible for additives where toxicity was manifest in

* Corresponding author. Tel.: +31 30 694 4545; fax: +31 30 6960264.
E-mail address: rob.stierum@tno.nl (R. Stierum).
[1] Present address: Bioinformatics and Genomics Department, Centro de Investigaciones Príncipe Felipe, Avda. Autopista Saler 16, 46012 Valencia, Spain.
[2] Present address: DSM Food Specialties R&D, Delft, The Netherlands.
[3] Present address: British American Tobacco, Group R&D Centre, Regents Park Road, Southampton, SO15 8TL, England.
[4] Present address: NV Organon, Part of Schering Plough Corporation, Oss, The Netherlands.

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the same target organ; the information on the additive had to indicate relevance of the effect for human health in terms of exposure levels; the uses and potential intakes of those additives which could not be excluded from showing joints actions or interactions on theoretical grounds. According to this criteria, possible joint actions or interactions could not be excluded for four food additives, with respect to the liver as target organ. All four compounds showed liver enlargement and hepatic hyperthrophy above the NOAEL in the subchronic oral toxicity study in the rat. These additives were butylated hydroxytoluene (BHT, E321), curcumin (CC, E100), propyl gallate (PG, E310) and thiabendazole (TB, E233) (Groten et al., 2000). Physiological effects of these compounds may be mediated through changes in the expression of gene products, either at the mRNA or protein level, or through interference with receptors. The current NOAEL however, used for these additives to perform risk assessment is only based upon conventional empirical endpoints such as relative liver weight, pathology and clinical chemistry. Some mechanistic information is available for the action of these food additives, often limited to the description of the effects of the compound on a small number of genes or proteins including drug metabolising enzymes (Groten et al., 2000). Little is known from a mechanistic perspective on how these food additives act in the liver at or below the NOAEL.

Since the past few years, novel genomics technologies are available which allow for the simultaneous determination of the expression of multiple genes at the mRNA (transcriptomics), protein (proteomics) and metabolite level (metabolomics) (Ball et al., 2002; Pandey and Mann, 2000; Schena et al., 1995). Knowledge on primary DNA sequence information together with knowledge on the transcriptome, proteome and metabolome constitutes the new area of genomics. Genomics approaches already have shown their value in toxicology and constitute the new research discipline of toxicogenomics (Anderson et al., 1987; Bushel et al., 2002; Hamadeh et al., 2002; Heijne et al., 2003; Stierum et al., 2005). Toxicogenomics enables the prediction of the mode of action and acquisition of mechanistic insights, when no or limited *a priori* knowledge is available on the compound under study.

To better understand the effects of BHT, CC, PG and TB at the molecular level, at first whenever administered as individual additives, a toxicogenomics analysis with the aim to find potential mechanism-based effect markers is employed. Here, gene expression changes in the liver together with additional supportive biochemistry are reported for these additives when administered individually for 28 days in the diet to male Sprague–Dawley rats.

2. Materials and methods

2.1. Materials

BHT (purity 99.9%), PG (purity 99.5%) and TB (purity 99.6%) were obtained from Sigma–Aldrich Company Ltd. (Poole, Dorset, UK). The supplier of CC (purity > 98%) was Acros Organics (Geel, Belgium). Unless otherwise indicated, all reagents were obtained from Sigma–Aldrich.

2.2. Preparation of diets and determination of dietary levels of additives

Desired target intakes of each of the additives were based on individual reports compiled by the JECFA as summarized by Groten et al. (2000) and other available data in recent literature. Ranges of target intakes were selected such that reported NOAELs and effect levels were included. Reported NOAELs and effect levels (in mg/kg/day) for the additives studied are: 25 and 100 (BHT); 220 and 440 (CC); 135 and 527 (PG); 10 and 37 (TB), respectively (Groten et al., 2000), corresponding to the intake of 254 and 1015 (BHT), 2234 and 4468 (CC), 1371 and 5351 (PG) and 102 and 376 (TB) ppm diets in this study. A single batch of fine ground rodent maintenance diet (R and M No. 1 diet) obtained from Special Diets Services (Witham, Essex, UK) was used for this study. BHT, CC, PG and TB were admixed with the fine ground diet using a mechanical mixer. For each of the four food additives, six samples from the lowest and highest dietary levels and two samples from the other three dietary levels were taken for analysis. Samples of diet were extracted with methanol and the filtered supernatant analysed by high performance liquid chromatography according to Price et al. (2004). Retention times of TB, PG, CC and BHT were 5.56, 6.96, 10.85 and 13.38 min, respectively, with the limits of quantification in rodent diet being 0.05, 0.1, 1.25 and 12.5 ppm, respectively. Diets were stored at 4 °C in closed containers and rodent food pots replenished at intervals of either 3 or 4 days. Analysis of samples of control diet demonstrated that the diet did not contain any significant levels of synthetic anti-oxidants, with apparent levels of BHT and PG being <4 and <0.7%, respectively, of the lowest dietary levels of these compounds used in this study. It should be noted that these values represent an overestimate of possible content of BHT and PG, as the peaks at these retention times contained a number of UV absorbing materials. Target and actual analysed levels of BHT, CC, PG and TB in the rodent diets are shown in Table 1.

2.3. Animals and treatment

Male Sprague–Dawley rats were obtained from Harlan Olac (Bicester, Oxon, UK). Animals were allowed free access to water and diets. The animals were kept in groups of 3 or 5 in polypropylene cages with stainless-steel grid tops and floors in rooms maintained at 22 ± 3 °C with a relative humidity of 40–70%. Rats were allowed to acclimatise to these conditions for six days before commencement of the study. Upon the start of the study rats were 7-weeks old and were either fed no test compound (control diet ($n = 10$)) or diets containing BHT, CC, PG or TB ($n = 6$ for each additive), during 28 days. Animal body weight and food consumption data were monitored for the duration of the study in order to calculate mean daily intakes for each dietary level of the four additives. At necropsy, rats were anaesthetised and killed by exsanguination. Blood was collected for clinical chemistry. Livers were immediately dissected, weighed and samples were taken for cDNA microarray analysis, biochemical analyses and histopathological evaluation. ~700 mg samples were taken from the left lobe, for cDNA microarray analysis. Samples (25–50 mg) were taken from the left lobe, flash frozen into liquid nitrogen for TaqMan® RT-PCR studies. Frozen liver samples were stored at −80 °C until further use.

2.4. Pathology

Samples of livers were preserved in a neutral aqueous phosphate-buffered 4 per cent solution of formaldehyde. The tissues to be examined microscopically were embedded in paraffin wax, sectioned at 5 μm and stained with haematoxilin and eosin. Histopathological examination was performed by means of light microscopy. Microscope slides were peer-reviewed.

2.5. Clinical chemistry

At necropsy, blood was collected from the abdominal aorta in heparinized plastic tubes and plasma was obtained by centrifugation. Measurements were performed on a Hitachi-911 Analyzer together with Boehringer reagents according to the manuals provided by the manufacturer. The following parameters were measured: alanine aminotransferase activity (ALT); albumin (ALB); aspartate aminotransferase activity (AST); cholesterol (CHOL); lactate dehydrogenase (LDH); sorbitol dehydrogenase (SDH), total protein (TP) and triglycerides (TRIG).

2.6. cDNA microarray experiments

Microarray experiments were performed in line with the Minimum Information About a Microarray Experiment (MIAME) principles (Brazma et al., 2001), as agreed upon by the Microarray Gene Expression Society. Further details are available at http://www.ebi.ac.uk/arrayexpress/ under Identifier E-MEXP-116. In summary, selection of liver samples for transcriptome analysis was made after completion of the histopathological analysis and clinical chemistry. Liver samples obtained from treatment groups with dose levels at or around the currently established NOAELs and higher were included. Thus, for CC and PG, microarray analysis was performed on samples obtained from animals from the three highest dose groups (CC: 2034, 4165 and 10,333 ppm; PG: 1464, 3280 and 6124 ppm). For BHT and TB, microarray analysis was performed on samples in those three highest dose groups were no changes in histology or clinical chemistry were observed (BHT: 744, 1457 and 2860 ppm; TB: 102, 240 and 758 ppm). Prior to isolation of total RNA, livers were homogenised with a mortar and pestle in liquid nitrogen. For each dose group, equal weight amounts (100 mg ± 5%) of liver were pooled from all animals within the same dose group (control group: 10 animals; compound groups: 6 animals). cDNA microarray analysis was performed using total RNA isolated from pooled liver material from animals within the same dose group. Gene expression studies using a pool of biological material have been performed before to study chemically-induced multiple gene expression changes. These studies show that transcriptome changes were comparable when cDNA microarray analysis was performed either on mRNA obtained from pooled liver from different animals versus mRNA obtained from one single animal (Bartosiewicz et al., 2001a, 2001b).

The cDNA array approach applied here, using Cy5 and Cy3 fluorophore labelling, does not measure the absolute amount of mRNA derived from each gene, but instead generates a relative ratio measurement of the experimental sample as compared to a reference sample. To allow for comparison of expression patterns