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Laurence Ribonnet, Edwige van Der Heiden, Ingrid Nobels, Agnès Chaumont, Anne-Sophie Remacle, Sarah de Saeger, Yves-Jacques Schneider, Marie-Louise Scippo, Ronny Blust, Luc Pussemier, et al.

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Laurence Ribonnet, Edwige van Der Heiden, Ingrid Nobels, Agnès Chaumont, Anne-Sophie Remacle, et al.. Potential of an *in vitro* toolbox combined with exposure data as a first step for the risk assessment of dietary chemical contaminants. Food Additives and Contaminants, 2011, pp.1. 10.1080/19440049.2011.584069 . hal-00719070

**HAL Id: hal-00719070**

**<https://hal.science/hal-00719070>**

Submitted on 19 Jul 2012

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**Potential of an in vitro toolbox combined with exposure data as a first step for the risk assessment of dietary chemical contaminants**

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2011-021.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	11-Apr-2011
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Methods/Techniques:	Bioassay, Receptors, Toxicology - cytotoxicity, Screening - microbial screening
Additives/Contaminants:	Mycotoxins, Pesticide residues
Food Types:	

Abstract:

In vitro risk assessment of dietary contaminants has become a priority in human food safety. In the present paper, we propose an in vitro approach associating different complementary tools in an original toolbox aiming at improving the assessment of the toxicological impact of dietary contaminants at realistic human exposure levels, with a special focus on the intestinal compartment. The system is based on the use of four complementary cellular tools, namely stress gene induction in transgenic strains of *Escherichia coli*, modulation of the activity of key biotransformation enzymes (cytochrome P-450 (CYP) 1A1 and 3A4) in a human intestinal cell line, and activation of aryl hydrocarbon receptor (AhR) and estrogenic receptor (ER)-dependent genes in agonistic and antagonistic assays with luciferase reporter cells. It was applied to four chosen model molecules: ochratoxin A (OTA) and deoxynivalenol (DON), two common food-borne mycotoxins, and imazalil (IMA) and benomyl (BEN), two fungicides widely recovered in foodstuffs. All these assays were performed at or around a realistic intestinal concentration, determined through a deterministic approach based on the calculation of a theoretical maximum daily intake (TMDI). Using the four model molecules, we clearly highlighted that induction of CYP1A1 activity and inhibition of CYP3A4 activity occurred in Caco-2 cells at a realistic intestinal concentration of IMA. Furthermore, some bacterial stress genes were induced in a range of realistic concentrations, following exposure to DON and IMA. In addition, BEN clearly provoked an ER agonistic activity in a human estrogen sensitive reporter cell line. All these results are in accordance with literature, suggesting that our in vitro toolbox constitutes an interesting approach in order to obtain a first 'fingerprint' of dietary contaminants at realistic human exposure for further risk assessment.

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1 **Potential of an *in vitro* toolbox combined with exposure data as a first**  
2 **step for the risk assessment of dietary chemical contaminants**

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20  
21 **Abstract**

22 *In vitro* risk assessment of dietary contaminants has become a priority in human food  
23 safety. In the present paper, we propose an *in vitro* approach associating different  
24 complementary tools in an original toolbox aiming at improving the assessment of the  
25 toxicological impact of dietary contaminants at realistic human exposure levels, with a  
26 special focus on the intestinal compartment. The system is based on the use of four  
27 complementary cellular tools, namely stress gene induction in transgenic strains of  
28 *Escherichia coli*, modulation of the activity of key biotransformation enzymes  
29 (cytochrome P-450 (CYP) 1A1 and 3A4) in a human intestinal cell line, and activation of  
30 aryl hydrocarbon receptor (AhR) and estrogenic receptor (ER)-dependent genes in

1 agonistic and antagonistic assays with luciferase reporter cells. It was applied to four  
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12 literature, suggesting that our *in vitro* toolbox constitutes an interesting approach in order  
13 to obtain a first 'fingerprint' of dietary contaminants at realistic human exposure for  
14 further risk assessment.

15

16 **Keywords:-** Food contaminants; intestine; stress genes; cytotoxicity; CYP; AhR;  
17 estrogen; Caco-2 cells; ochratoxin A; deoxynivalenol; imazalil; benomyl

18

## 19 **Introduction**

20 Nowadays, the chemical safety of food is of major concern since our diet contains a wide  
21 range of undesired substances. These food contaminants include for example pesticides,  
22 heavy metals and persistent organic pollutants (POPs), which are widespread in the  
23 environment and readily enter the food chain. In addition, food contamination may also  
24 occur during food processing and cooking (*e.g.* benzo[*a*]pyrene (B[*a*]P), acrylamide) or  
25 through the release of chemicals from consumer products, such as baby toys, and  
26 packaging (*e.g.* bisphenol A, phthalates). Furthermore, mycotoxins, secondary  
27 metabolites produced by fungal species in high moisture conditions occurring in field or  
28 during storage of cereals or other agricultural goods, are recovered in derived foodstuffs.  
29 Due to the widespread of dietary contamination by mycotoxins, these substances are also  
30 considered as an important hazard for human and animal health.

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1 Given the fact that the effects of food contaminants on human health are not always  
2 thoroughly known, more investigations about their toxicity and more risk assessment  
3 studies should be performed. The process of risk assessment generally integrates  
4 information from both exposure assessment and hazard characterization. In order to be  
5 relevant, the hazard must be characterized experimentally at realistic doses.

6  
7 The dietary contaminants present in the gastro-intestinal tract should gain access to the  
8 intestinal mucosa. There, the enterocytes, can be considered on one hand as the first  
9 biological barrier encountered by the xenobiotics and, on the other hand, as a human  
10 tissue submitted to high concentrations of these compounds. The large microbial  
11 population present in the intestine and especially in the colon (*e.g. Bacteroides*,  
12 *Lactobacillus*, *Bifidobacterium*, *Escherichia*) should also be submitted to most of the  
13 dietary contaminants and could be affected. These microbes contribute to the normal  
14 digestive function and, in some cases, to the transformation of dietary compounds or  
15 drugs into often more readily absorbed and more or less toxic forms (Sousa *et al.*, 2008;  
16 Cani and Delzenne, 2009; Laparra and Sanz, 2010). Toxicity on bacteria may thus have  
17 an impact on the bioavailability and effects of ingested chemical compounds.

18  
19 In order to estimate realistic concentrations at the intestinal level, one has to take into  
20 account the dilution of the contaminant in the alimentary bolus. In addition, the question  
21 of bioaccessibility should ideally be considered as well. For assessing realistic  
22 concentrations at the hepatic level, absorption and intestinal first-pass metabolism of  
23 dietary compounds are also of crucial importance. Recommendations for revising current  
24 risk assessment models are often stated, with the objective to reduce animal testing, to  
25 improve relevance of toxicodynamic data for human health and to focus on realistic  
26 exposure and toxicokinetics. Consistent with these recommendations, a package of high  
27 throughput *in vitro* experimental tools would be necessary in order to quickly assess some  
28 important toxicological effects due to the dietary contaminants at realistic exposure  
29 levels. In this paper, we therefore elaborated a model toolbox comprising a set of selected  
30 complementary relevant *in vitro* assays in bacterial and eukaryotic cell types, allowing

1 taking a first 'fingerprint' of tested toxicants in realistic chronic exposure conditions, with  
2 a special focus on the intestinal compartment.

3  
4 In a first *in vitro* assay, we used reporter genes in genetically modified *Escherichia coli*  
5 strains that respond to different types of stress (Dardenne *et al.*, 2007; Dardenne *et al.*,  
6 2008), in order to assess acute toxicity and give an insight into the toxic mode of action  
7 of dietary compounds.

8  
9 A second *in vitro* assay focused on the cytochrome P-450 (CYP) biotransformation  
10 enzymes present in enterocytes. These cells are indeed the first major site for metabolism  
11 prior to systemic uptake of orally ingested xenobiotics and the major catalysts of first-  
12 pass metabolism in enterocytes are the CYP biotransformation enzymes, mainly the  
13 CYP3A4 isoform and to a lesser extent, the CYP1A1 isoform (Prueksaritanont *et al.*,  
14 1996; Lown *et al.*, 1997; Lampen *et al.*, 1998; Paine *et al.*, 1999; Zhang *et al.*, 1999;  
15 Paine *et al.*, 2006). However, a great inter-individual variability in the expression of these  
16 CYP isoforms may be observed in the human intestine (Paine *et al.*, 1997; Paine *et al.*,  
17 1999; Lindell *et al.*, 2003; Paine *et al.*, 2006). It has been established in enterocytes as  
18 well as in the Caco-2 intestinal cell line, that CYP1A1 is likely to be induced rather than  
19 constitutive (Boulenc *et al.*, 1992; Rosenberg and Leff, 1993; Buchthal *et al.*, 1995;  
20 Prueksaritanont *et al.*, 1996), with diet clearly taking part in this induction (Fontana *et al.*,  
21 1999). Although most CYP oxidations are involved in detoxification pathways, CYP1A1  
22 also catalyzes the production of reactive cytotoxic metabolites and the activation of pro-  
23 carcinogens, such as PAHs, like B[a]P, into carcinogenic DNA-binding species (Schwarz  
24 *et al.*, 2001; Shimada *et al.*, 2001). Furthermore, some endogenous compounds are  
25 metabolized by CYP1A1. It has been shown, for example, that CYP1A1 is implicated in  
26 all-*trans*-retinoic acid (*atRA*) metabolism (Lampen *et al.*, 2000; Marill *et al.*, 2000;  
27 McSorley and Daly, 2000) and 17 $\beta$ -estradiol (E2) catabolism to weak estrogen (Lee *et*  
28 *al.*, 2003; Tsuchiya *et al.*, 2005). Therefore, dietary regulation of CYP enzymes may  
29 contribute to pathogenic mechanisms (Murray, 2006). It is also well known that CYP3A4  
30 is implicated in the metabolic processing of a large range of (pro-)drugs. The co-  
31 occurrence at the level of the intestinal epithelium of other compounds modulating the

1 CYP3A4 content, such as grapefruit juice, may be detrimental (Guengerich, 1997; Lown  
2 *et al.*, 1997; Dresser *et al.*, 2000).

3  
4 The third *in vitro* assay focused on the aryl hydrocarbon nuclear receptor (AhR), a ligand-  
5 activated transcription factor of the Per-Arnt-Sim (PAS) superfamily, which is known to  
6 regulate CYP1A1 transcription. It is located in its free state in the cytosol, as a complex  
7 with the chaperone proteins Hsp90, p23 and immunophilins. Ligand binding to AhR  
8 triggers its nuclear translocation and fixation to the aryl receptor nuclear translocator  
9 (Arnt), resulting in binding of the complex to the dioxin-responsive elements (DREs) on  
10 target genes (Denison and Whitlock, 1995; Delescluse *et al.*, 2000; Ma, 2001; Mimura  
11 and Fujii-Kuriyama, 2003; Kawajiri and Fujii-Kuriyama, 2007). Several very toxic  
12 dietary contaminants, like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), have been  
13 reported to bind to AhR and to trigger downstream target gene transcription (Denison and  
14 Nagy, 2003; Ashida *et al.*, 2008). It is now well established that, among other genes, the  
15 transcription of CYP1A1, CYP1A2, UDP-glucuronosyltransferase (UGT) and  
16 glutathione-*S*-transferase (GST) is mediated by the AhR.

17  
18 A fourth and last *in vitro* assay focused on the estrogen receptor (ER), another ligand-  
19 activated transcription factor, belonging to the superfamily of steroid hormone nuclear  
20 receptors. ER needs ligand binding in the nucleus to trigger conformational changes and  
21 to subsequently homodimerize, bind to an estrogen response element (ERE) and activate  
22 transcription of its target genes (Nilsson *et al.*, 2001). Furthermore, a cross-talk between  
23 AhR and ER pathways has been established on the basis of anti-estrogenic effects of AhR  
24 ligands, such as TCDD (Nilsson *et al.*, 2001; Ohtake *et al.*, 2008; Ohtake *et al.*, 2009).  
25 Since (i) the toxic effects of TCDD are known to be in part mediated by the AhR  
26 (Mimura and Fujii-Kuriyama, 2003; Bock and Köhle, 2006), (ii) an estrogenic disruption  
27 may arise from exogenous ligand binding on ER (le Maire *et al.*, 2010), (iii) the ligand-  
28 activated AhR complex is able to potentiate or repress either DRE or ERE target genes  
29 through association with ER complex by cross-talk mechanisms (Nilsson *et al.*, 2001;  
30 Ohtake *et al.*, 2008; Ohtake *et al.*, 2009), (iv) the human diet contains a large amount of



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2  
3 1 AhR/ER-activating substances, attention should be paid to molecules able to act on the  
4  
5 2 AhR/ER pathways.  
6

7 3  
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9 4 Considered together, the four *in vitro* experimental tools described above constitute a  
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11 5 package of complementary tests that may be considered as an efficient toolbox allowing  
12  
13 6 taking a first 'fingerprint' of potential toxicants. The aim of the present work was to  
14  
15 7 assess the pertinence of this toolbox by applying it to four well-known dietary  
16  
17 8 contaminants displaying distinct toxicological effects and showing human health concern:  
18  
19 9 two mycotoxins, namely ochratoxin A (OTA) and deoxynivalenol (DON), and two  
20  
21 10 fungicides, imazalil (IMA) and benomyl (BEN). The chemical structures of these  
22  
23 11 compounds are shown in Figure 1.  
24

25 12  
26 13 OTA is produced by fungal species from the genera *Penicillium* and *Aspergillus* and  
27  
28 14 occurs in cereals, but also in nuts, pulses, beans, spices, dried fruits, grape(s) (juice),  
29  
30 15 wine, raisins, beer, cocoa products or coffee (EFSA, 2006). Furthermore, the carry-over  
31  
32 16 to animal offal might be important, especially in the kidney for which however the human  
33  
34 17 consumption is relatively limited (EFSA, 2004). It is mainly nephrotoxic in mammalian  
35  
36 18 species, but also immunotoxic, neurotoxic and teratogenic (EFSA, 2006). The occurrence  
37  
38 19 of OTA in food and human blood samples has been associated with human endemic  
39  
40 20 nephropathy and tumours of the upper urinary tract, in different countries (Maaroufi *et*  
41  
42 21 *al.*, 1995; Radic *et al.*, 1997; Stoev, 1998; Vrabcheva *et al.*, 2000). However, due to  
43  
44 22 inadequate evidence in humans, OTA has been classified as possible human carcinogen  
45  
46 23 (group 2B) by the International Agency for Research on Cancer (IARC, 1993). One  
47  
48 24 debated hypothesis for the carcinogenicity of OTA in kidney implies covalent DNA  
49  
50 25 adduct formation after bioactivation by biotransformation enzymes (for a review, see  
51  
52 26 Pfohl-Leszkowicz and Manderville (2007)). DON is a trichothecene also called  
53  
54 27 vomitoxin, which is produced by strains of *Fusarium*. It is often reported in wheat,  
55  
56 28 barley, maize, oat, rice, rye, sorghum and triticale but also in processed food products  
57  
58 29 such as wheat flour, bread, beer or noodles. In humans, primary targets of DON are the  
59  
60 30 immune function (Pestka and Smolinski, 2005) and the mucosa of the gastro-intestinal  
31  
32 31 tract, which may be damaged, often resulting in gastroenteritis symptoms after acute

1 exposure. Furthermore, genotoxic potential of DON and association with esophageal  
2 cancer have been suggested (Rotter *et al.*, 1996; Bony *et al.*, 2006).

3  
4 Pesticides may also be daily ingested, mainly through fruit and vegetable consumption.  
5 IMA is a post-harvest imidazole fungicide mainly applied on citrus fruits. This chemical  
6 is also used as broad-spectrum veterinary topical antimycotic agent under the name of  
7 enilconazole (Vanden Bossche *et al.*, 2003). IMA belongs to the group of the 10  
8 pesticides the most frequently reported in fruits and vegetables by European Member  
9 States (Poulsen and Andersen, 2003). The mode of action of IMA is based on the  
10 inhibition of sterol demethylation, which affects membrane integrity in fungal cells  
11 (Ghannoum and Rice, 1999; EFSA, 2007). Little information is available regarding the  
12 toxic effects of IMA on human health. However, it seems that the liver is the primary  
13 target for IMA in experimental animals (EFSA, 2007) even if IMA has also been shown  
14 to act on cytochrome P450 (CYP) activity in the intestinal Caco-2 cell line (Sergent *et al.*,  
15 2009). IMA is an inhibitor of the activity of human aromatase (CYP19), an enzyme that  
16 catalyzes the conversion of androgens to estrogens; it appears to be as potent as the  
17 aromatase inhibiting antiestrogen drugs used to treat breast cancer (Vinggaard *et al.*,  
18 2000; McKinlay *et al.*, 2008). BEN is another widely used fungicide, belonging to the  
19 imidazole family. Residues of carbendazim and *n*-butylisocyanate, two degradation  
20 products of BEN in soils and aqueous solutions, are commonly reported in fruits and  
21 vegetables. Hepatotoxicity, teratogenicity, male reproductive toxicity and carcinogenicity  
22 have been reported following exposure to BEN (Igbedioh and Akinyele, 1992; McCarroll  
23 *et al.*, 2002; Hewitt *et al.*, 2005).

24  
25 Practically, the proposed *in vitro* toolbox to be evaluated has been applied through the  
26 following procedure to OTA, DON, IMA and BEN toxicity assessment at dietary realistic  
27 exposure levels. At first, realistic concentrations of these contaminants occurring in the  
28 gastro-intestinal tract have been calculated and cytotoxicity assessed. Secondly, the  
29 effects of OTA, DON, IMA and BEN on stress genes from an intestinal bacterium and on  
30 the activity of key intestinal biotransformation enzymes (CYP1A1 and CYP3A4) have  
31 been investigated at realistic experimental concentrations. Since induction of CYP1A1

1 activity is mainly mediated by the AhR pathway and considering that some toxic effects  
2 may be mediated by this receptor (*e.g.* TCDD), we also investigated dose-dependent AhR  
3 agonistic and antagonistic activities in luciferase reporter cells. Furthermore, still using  
4 luciferase reporter cells, we assessed the dose-dependent agonistic and antagonistic  
5 activities on the human estrogenic receptor (hER), in order to detect potential estrogenic  
6 disruption by dietary contaminants mimicking estrogens. Results were systematically  
7 compared with the existing literature in the discussion part, in order to validate our  
8 toolbox and to propose it as an efficient tool for the screening of toxicological properties  
9 of less studied dietary contaminants.

## 11 **Materials and methods**

### 12 *Determination of realistic intestinal concentrations*

13 In order to work with realistic intestinal concentrations, we have considered that the daily  
14 intake of a contaminant, which is the sum of the quantity ingested through the  
15 consumption of the different foodstuffs containing the substance, is diluted in the  
16 alimentary bolus. This quantity ingested is obtained through the multiplication of  
17 consumption and contamination data for each foodstuff susceptible to contain the  
18 substance.

19  
20 In the present work, we have used European consumption data from the GEMS/FOOD  
21 Regional Diets (WHO, 2003). These diets, based on food balance sheet data compiled by  
22 the FAO, give regional *per capita* average consumption [g/person/day] of raw and semi-  
23 processed agricultural commodities. We have also used the European maximum (residue)  
24 limits (M(R)Ls) for both mycotoxins (Commission Regulation (EC) N°1881/2006 of 19  
25 December 2006) and pesticides (Commission Regulation (EC) N°396/2005 of 23  
26 February 2005, amended by Commission Regulation (EC) N°839/2008 of 31 July 2008  
27 for BEN and Commission Regulation (EU) N°750/2010 of 7 July 2010 for IMA) in the  
28 main consumed foodstuffs as contamination data. We have then applied the concept of  
29 theoretical maximum daily intake (TMDI), which assumes that a dietary chemical occurs  
30 in food at the M(R)L legally authorized and that the average daily consumption of  
31 foodstuffs per person corresponds to the published values from GEMS/FOOD Regional

Diets. The daily intake estimated through that approach is representative of a long-term exposure (WHO, 1997) (Formula 1).

$$TMDI [ng/person/day] = \sum_i (Q_{E_i} \cdot M(R)L_i)$$

- $Q_{E_i}$ : consumed quantity of foodstuff  $i$  in the European diet from GEMS/FOOD Regional Diets [g/person/day]

- $M(R)L_i$ : Maximum (residue) limit of contaminant in foodstuff  $i$  [ $\mu$ g/kg]

**Formula 1: Theoretical maximum daily intake (TMDI) of a dietary chemical contaminant**

Dilution hypothesis of the calculated daily intake is based on the assumption that, in the worst case, the dilution occurs in 1L of gastro-intestinal secretions per meal, with one meal per day and gives a maximal realistic intestinal concentration (C) (Formula 2).

$$C [ng/ml] = \frac{TMDI}{V \cdot n \cdot 1000}$$

- TMDI: theoretical maximum daily intake [ng/person/day]

- V: volume of gastro-intestinal fluid [L/meal] ( $V = 1L$ )

- n: number of daily meals [meals/person/day] ( $n = 1$ )

**Formula 2: Maximal realistic intestinal concentration (C)**

Thanks to this method, we obtained a maximal realistic intestinal concentration (C) representative of chronic exposure. The concentration or concentration range of OTA, DON, IMA or BEN used in cellular assays were chosen to be close to or to include the calculated realistic intestinal concentration of the relevant contaminant.

**Chemical contaminants**

BEN (95%), DON (> 97%), IMA (> 97%), OTA (> 98%) and B[a]P were purchased from Sigma-Aldrich (St Louis, MO). TCDD (99.9%) was supplied from BCP Instruments, Wellington Laboratories Inc. (Guelph, CA).

**Cell culture**

Caco-2 cells

Caco-2 cells (ATCC, Rockville, MD), derived from a human colon adenocarcinoma, were used between passages 26 and 34 and routinely grown in Dulbecco's Modified

1 Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% heat-  
2 inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Waltham, MA), 0.1  
3 mM non-essential amino acids (Lonza, Walkersville, MD) and 2mM L-glutamine  
4 (Lonza). Cells were incubated at 37°C in a humidified chamber with 5% CO<sub>2</sub>, with  
5 medium change every 2 days.

#### 6 Stable reporter cell lines

7 Rat hepatoma H4IIE (DR-CALUX<sup>®</sup>) (Biodetection System, NL), and human hepatoma  
8 HepG2-Luc were used as dioxin sensitive reporter cells containing AhR, and human  
9 breast cancer MMV-Luc cells (originating from MCF-7 cells) as estrogen sensitive  
10 reporter cells containing mainly ER- $\alpha$ . These three cell lines were the result of a stable  
11 transformation with an inducible firefly luciferase reporter gene (Aarts *et al.*, 1993;  
12 Willemsen *et al.*, 2004; Van der Heiden *et al.*, 2009). H4IIE (DR-CALUX<sup>®</sup>), HepG2-Luc  
13 and MMV-Luc cells were grown respectively in Minimum Essential Medium (MEM)  
14 alpha, MEM and DMEM (all from Thermo Fisher Scientific Inc.), supplemented with  
15 10% heat-inactivated FBS (AE scientific, Marcq, BE). DMEM was further supplemented  
16 with 100 U/ml of penicillin G, 100  $\mu$ g/ml of streptomycin (Thermo Fisher Scientific Inc.)  
17 and 1  $\mu$ g/ml of bovine pancreas insulin (Sigma-Aldrich). Cells were grown at 37°C in 5%  
18 CO<sub>2</sub>.

#### 19 Bacterial strains

20 All strains of *E. coli*, a common Proteobacterium from the human gut microbiota, used in  
21 the present study (KatG, Zwf, Soi28, Nfo, MicF, OsmY, UspA, ClpB, MerR, RecA,  
22 UmuDC, Ada, SfiA and DinD), except SfiA, are based on an *E. coli* K-12 derivative SF1  
23 containing the mutations *lac4169* deleting the entire *lac* operon, and *rpsL*. All the  
24 different *LacZ* fusions are present as single chromosomal inserts. The strains, described  
25 in Dardenne *et al.* (2007), were selected from the work of Orser *et al.* (1995) and respond  
26 to different types of stress: DNA damage, oxidative stress, protein denaturation,  
27 membrane damage, osmotic stress, general cellular stress and heavy metal presence. The  
28 SfiA strain is part of the SOS chromotest derived from *E. coli* GC4436 with a deletion in  
29 the *lac* operon carrying a *sfiA::lacZ* fusion, so that responses to DNA damaging agents  
30 can be measured (Quillardet and Hofnung, 1985).

31

## 1 *Cytotoxicity*

2 Cytotoxicity was assessed in the different eukaryotic cell lines. Colorimetric assays were  
3 performed and cell morphology was routinely observed by phase contrast microscopy.

### 4 Caco-2 cells

5 The colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-  
6 sulfophenyl)-2H-tetrazolium (MTS) assay purchased as a kit (Celltiter 96<sup>®</sup> AQueous One  
7 Solution Cell Proliferation Assay, Promega, Madison, WI) was used. It measures cellular  
8 metabolic activity as a way to assess the effect of dietary contaminants on Caco-2 cell  
9 proliferation. Cells were seeded in 96-well plates at a density of  $1.10^4$  cells/cm<sup>2</sup> and were  
10 incubated during 48h, 24h after seeding, with OTA, DON, IMA or BEN diluted in 0.5%  
11 FBS-enriched culture medium at a final concentration of respectively 1 ng/ml, 250 ng/ml,  
12 2500 ng/ml and 250 ng/ml. Control vehicles corresponding to 0.0002% (v/v) DMSO for  
13 OTA, 0.05% (v/v) ethanol for DON, and 0.01% (v/v) DMSO for IMA and BEN as well  
14 as negative control, consisting in 0.5% FBS-enriched culture medium alone, were also  
15 tested. The MTS assay was carried out as described by the manufacturer with formazan  
16 production detected at 500 nm (reference wavelength: 690 nm) with a Spectracount  
17 spectrophotometer (Packard, Warrenville, IL).

18  
19 As another indicator of cytotoxicity, the release of cytosolic lactate dehydrogenase  
20 (LDH) by damaged and necrosed cells into the extracellular medium was measured in the  
21 same conditions as for the EROD assay (see below). Caco-2 cells were seeded in 48-well  
22 plates at a density of  $3.10^4$  cells/cm<sup>2</sup> and were cultivated until 16-day post-confluence  
23 with culture medium changes three times per week. Cells were then incubated for 6 or  
24 24h with OTA, DON, IMA, BEN or vehicles diluted in FBS-free culture medium at the  
25 same final concentration as for the MTS assay. The negative control consisted in FBS-  
26 free culture medium alone and the positive control, corresponding to 100% of cell  
27 mortality, consisted in cells treated with 1% (v/v) Triton<sup>®</sup> X-100 (Sigma-Aldrich) in  
28 FBS-free culture medium. The activity of LDH in the medium was determined with the  
29 Cytotoxicity detection kit (Roche diagnostics, Mannheim, DE) according to the  
30 manufacturer's instructions with formazan production detected at 500 nm (reference  
31 wavelength: 690 nm) with a Spectracount spectrophotometer (Packard).

### 1 Stable reporter cells

2 The assay routinely used to test the effect of the selected chemicals on the proliferation of  
3 stable reporter cells was the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium  
4 bromide (MTT) colorimetric assay, which also permits to measure cellular metabolic  
5 activity. H4IIE DR-CALUX<sup>®</sup>, HepG2-Luc and MMV-Luc cells were seeded in 96-well  
6 plates at a density of respectively 94000, 31000 and 62500 cells/cm<sup>2</sup>, in phenol red-free  
7 DMEM supplemented with 10% heat-inactivated FBS for both H4IIE and HepG2-Luc  
8 cell lines and with 10% charcoal-dextran-treated FBS, aiming at eliminating potential  
9 hormones that could interfere during the assay, for the MMV-Luc cell line. MMV-Luc  
10 cells were put in contact with the charcoal-dextran-treated FBS-enriched culture medium  
11 24h before seeding. After overnight incubation, cells were exposed during 24h to serial  
12 dilutions of OTA, DON, IMA or BEN, to culture medium alone, or to culture medium  
13 containing 0.4% (v/v) of the vehicle for all tested concentrations (ethanol for OTA and  
14 IMA, water for DON and DMSO for BEN). Final concentrations used for the three cell  
15 lines were as follows. OTA: 4, 40, 400, 4000 and 40000 ng/ml. DON: 3.2, 16, 80, 400,  
16 and 2000 ng/ml. BEN: 64, 320, 1600, 8000, and 40000 ng/ml. For IMA, two different  
17 ranges of concentration were used: 312, 625, 1250, 2500, 5000, 10000, 20000, and 40000  
18 ng/ml with both the H4IIE DR-CALUX<sup>®</sup> and HepG2-Luc cell lines and 16, 125, 250,  
19 500, and 4000 ng/ml with the MMV-Luc cell line. After medium removal, cells were  
20 washed with 100 µl phosphate buffered saline (PBS) (Oxoïd, Hampshire, UK) and  
21 incubated at 37°C with 100 µl yellow MTT reagent (0.5 mg/ml) (Sigma-Aldrich) during  
22 3h. The colored insoluble formazan reagent produced by metabolically active  
23 mitochondria was made soluble with DMSO and detected at 540 nm (reference  
24 wavelength: 620 nm) with a Multiskan Ascent spectrophotometer (Thermo Fisher  
25 Scientific Inc.) after plate agitation during 15 min.

### 27 ***Bacterial stress gene profiling assay***

28 The entire procedure for the bacterial stress gene profiling assay is described in Dardenne  
29 *et al.* (2007). The different *E. coli* strains were exposed for 90 min in the exponential  
30 growth phase to a ½ serial dilution of OTA (1.6 – 100 µg/ml), DON (0.031 - 2 µg/ml),  
31 IMA (0.4 - 13 µg/ml) or BEN (0.78 – 50 µg/ml) or to vehicle alone, corresponding to 1%

(v/v) methanol for OTA and DON and 1% (v/v) DMSO for IMA and BEN, for all tested concentrations. Afterwards, the cells were lysed and the  $\beta$ -galactosidase activity was measured.

Activity of the promoter is calculated taking into account the growth inhibition of the used strain. The results are presented as fold inductions at a given dose  $i$  relative to the control values and were calculated through a set of formulas (Formula 3 and 4).

$$Activity_i = 0,19 \times \left( \frac{OD_{420nm}^{PE} - OD_{420nm}^{SE}}{(OD_{600nm}^{PD} \times 90\text{min}) + ((OD_{600nm}^{PE} - OD_{600nm}^{SE}) \times \frac{90\text{min}}{2})} \right)$$

**Formula 3: Activity at a given dose  $i$ .** PE, Post exposure; SE, start exposure (=post dose); PD, pre-dose (Dardenne *et al.*, 2007).

$$Fold\_Induction = \frac{Activity_i}{Average\_Activity_{negative\_controls}}$$

**Formula 4: Fold Induction at a given dose  $i$  (Dardenne *et al.*, 2007).**

## 2.6 Modulation of intestinal cytochrome P-450 biotransformation enzyme activity

### 7-Ethoxyresorufin-O-deethylase (EROD) activity assay

The measurement of the EROD activity allowed us to assess the effects of the tested compounds on the catalytic activity of CYP1A1 in enterocytes. Caco-2 cells were inoculated at  $3.10^4$  cells/cm<sup>2</sup> and cultivated until 16 days post-confluence (time of maximal inducibility by B[a]P in our experimental conditions) in 48-well plates with culture medium changes 3 times per week. Cells were then incubated for 6 or 24h with OTA, DON, IMA or BEN diluted in FBS-free culture medium at a final concentration of respectively 1 ng/ml, 250 ng/ml, 2500 ng/ml and 250 ng/ml. Control vehicles corresponding to 0.0002% (v/v) DMSO for OTA, 0.05% (v/v) ethanol for DON, and 0.01% (v/v) DMSO for IMA and BEN were also tested. Positive controls consisted in B[a]P (250 ng/ml) or TCDD (0.3 ng/ml) and negative control consisted in FBS-free culture medium alone (untreated cells). Cells were rinsed with Hank's Balanced Salt Solution (HBSS) (Lonza) and incubated for 30 minutes at 37°C with 300  $\mu$ l of 7-ethoxyresorufin (5  $\mu$ M) (Sigma-Aldrich) in phenol red-free DMEM (Lonza), in the absence of contaminants or vehicles. Fluorescence due to the conversion of the substrate



1 to resorufin was measured, in the supernatants, using a Fluoroskan Ascent FL fluorimeter  
2 (Thermo Fisher Scientific Inc.) with excitation and emission wavelengths of 530 and 585  
3 nm, respectively. The EROD activity was normalized to the cell protein content  
4 determined with the bicinchoninic acid protein (BCA) assay kit (Sigma–Aldrich) after  
5 cell lysis with 1% (w/v) sodium deoxycholate (DOC) in water, at pH 11.3.

#### 6 CYP3A4 activity assay

7 The CYP3A4 activity assay was used to estimate the inhibitory effect of the tested  
8 compounds on the catalytic activity of CYP3A4 in enterocytes. Caco-2 cells were seeded  
9 at  $4.10^4$  cells/cm<sup>2</sup> in 12-well plates pre-coated with type I collagen (Sigma-Aldrich) and  
10 grown to confluence. As described by Schmiedlin-Ren *et al.* (1997), Caco-2 cells were  
11 then cultivated during 2 weeks in 5% FBS-enriched DMEM and in the presence of 0.5  
12  $\mu$ M of 1, $\alpha$ 25-(OH)<sub>2</sub>-vitamin D<sub>3</sub> (1,25-vitD<sub>3</sub>) (Sigma-Aldrich), in order to express  
13 functional CYP3A4 activity, with culture medium changes three times per week. The last  
14 day, the cells were rinsed with PBS and incubated for 3h at 37°C with 500  $\mu$ M  
15 testosterone, in the absence of other compound (negative control) or in the presence of  
16 ketoconazole (positive control), OTA, DON, IMA or BEN diluted in Hanks' balanced  
17 salt solution (HBSS) at pH 7.4 at a final concentration of respectively 26.6  $\mu$ g/ml (50  
18  $\mu$ M), 1 ng/ml, 250 ng/ml, 2500 ng/ml and 250 ng/ml. Supernatants were then collected  
19 for 6 $\beta$ -(OH)-testosterone quantification through HPLC, as previously described in  
20 Sergeant *et al.* (2009). The limits of detection (LOD) and quantification (LOQ) were 100  
21 and 300 nM, respectively. The CYP3A4 activity was normalized to the cell protein  
22 content determined with the BCA assay kit (Sigma–Aldrich) after cell lysis with 1%  
23 (w/v) DOC in water, pH 11.3.

#### 25 **Human and rat aryl hydrocarbon receptor (AhR) agonistic/antagonistic activities**

26 The cell-based assays to screen for agonistic/antagonistic AhR activities of chemicals  
27 were carried out as previously described (Van der Heiden *et al.*, 2009). Briefly, 94000  
28 cells/cm<sup>2</sup> were seeded in 96-well culture plates and incubated overnight. In assays to  
29 measure AhR agonistic activities, H4IIE DR-CALUX<sup>®</sup> or HepG2-Luc cells were exposed  
30 during 6 or 24h to serial dilutions of OTA, DON, IMA or BEN, at the same final  
31 concentrations as for the cytotoxicity assay, or to culture medium containing 0.4% (v/v)

1 of the vehicle for all tested concentrations (ethanol for OTA and IMA, water for DON  
2 and DMSO for BEN). In assays to measure AhR antagonistic activities, the cells were  
3 exposed during 24h simultaneously to a constant concentration of the AhR inducer  
4 TCDD (0.1 or 3 ng/ml, for H4IIE DR-CALUX<sup>®</sup> and HepG2-Luc, respectively) and to  
5 increasing concentrations of OTA, DON, IMA or BEN (the same range as for the  
6 agonistic assay). The inducer concentration was chosen to obtain a maximal luciferase  
7 activity. Solvent concentration was 0.8% (v/v) in the culture medium for all tested  
8 concentrations. In both assays (agonistic and antagonistic activities), the medium was  
9 removed after incubation with the target analytes and the cells were lysed with 50 µl of a  
10 solution containing 25 mM Tris, 2 mM 1,4-dithio-DL-thréitol (DTT), 2 mM trans-1,2-  
11 Diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate (DCTA), 10% (v/v)  
12 glycerol and 1% (v/v) Triton X-100 (all from Sigma–Aldrich). An ORION II  
13 luminometer equipped of an automatic injector (Berthold Detection System, Pforzheim,  
14 DE) was used to add 100 µl of a solution containing both luciferin (0.15 mg/ml)  
15 (Synchem OHG, Felsberg/Altenburg, DE) and ATP (0.3 mg/l) (Roche Diagnostics), and  
16 to directly measure the emission of light resulting from the luciferase activity and  
17 reported as Relative Light Units (RLUs).

18  
19 In assays to measure AhR agonistic activities, when a dose-response curve was obtained,  
20 the median effective concentration (EC<sub>50</sub>) was calculated from a non linear equation  
21 (Formula 5). The relative potency (REP) of a chemical was calculated as the ratio  
22 between the EC<sub>50</sub> of the reference ligand and that of the chemical. Antagonistic activity  
23 was expressed as a relative response, *i.e.* in percentage of the maximal cell response to  
24 TCDD, corresponding to 100%.

$$y = \frac{A_0}{1 + \left(\frac{x}{A_1}\right)^{A_2}}$$

25  
26 **Formula 5: Non linear equation of a dose-response curve allowing determining**  
27 **EC<sub>50</sub>.** y is cell response, expressed in RLUs or in relative response (%); x is the analyte  
28 concentration; A<sub>0</sub> is the maximal response expressed in RLUs or in relative response (%);  
29 A<sub>1</sub> is EC<sub>50</sub> and A<sub>2</sub> is the slope of the linear part of the curve.

### 1 ***Human estrogen receptor (hER) agonistic/antagonistic activities***

2 In order to test agonistic/antagonistic hER activities of dietary contaminants, MMV-Luc  
3 cells were used. These cells were put in contact with the charcoal-dextran-treated FBS-  
4 enriched culture medium 24h before seeding, as explained previously for the cytotoxicity  
5 assays. MMV-Luc cells were then seeded in 96-well plates at a density of 22000  
6 cells/cm<sup>2</sup> and incubated overnight. In assays to measure hER agonistic activities, MMV-  
7 Luc cells were exposed during 24h to serial dilutions of OTA, DON, IMA or BEN, at the  
8 same final concentrations as for the cytotoxicity assay, or to the culture medium  
9 containing 0.4% (v/v) of the vehicle for all tested concentrations (ethanol for OTA and  
10 IMA, water for DON and DMSO for BEN). In assays to measure hER antagonistic  
11 activities, the cells were exposed during 24h simultaneously to a constant concentration  
12 of the hER inducer E2 (1.4 ng/ml) (Sigma-Aldrich) and to increasing concentrations of  
13 OTA, DON, IMA or BEN (the same range as for the agonistic assay), or to 60.7 ng/ml  
14 (100 nM) of fulvestrant (Sigma-Aldrich) as a positive antagonistic control (Osborne *et*  
15 *al.*, 2004). The inducer concentration was chosen to obtain a maximal luciferase activity,  
16 which was set at 100%. Solvent concentration was 0.8% (v/v) in the culture medium for  
17 all tested concentrations.

18 The emission of light resulting from the luciferase activity was measured and data were  
19 analyzed and expressed as previously described for the AhR assay.

### 20 ***Statistical analysis***

21 Results were expressed in means  $\pm$  S.E.M. of three independent experiments performed  
22 in triplicate (n=9). Statistical analysis was conducted with the SPSS 18.0 computer  
23 program (IBM, Somers, NY), using one-way analysis of variance (ANOVA) coupled  
24 with post-hoc test of Scheffe or Tukey. When comparison was made with a reference  
25 group, post-hoc test of Dunnett was used. When homogeneity of variance was not  
26 assumed, the correction of Welch was applied and coupled with the post-hoc test of  
27 Dunnett T3. Homogeneity of variance was verified with the Levene statistic. A log  
28 transformation was performed to achieve normality when necessary. Significant effects  
29 were determined in comparison with respective vehicle conditions.

30  
31

## 1 Results

### 2 *Determination of realistic intestinal concentrations*

3 European dietary consumption data from GEMS/FOOD Regional Diets (WHO, 2003)  
4 and European M(R)Ls of contaminants legally authorized in the main consumed  
5 foodstuffs have allowed us to calculate TMDIs for OTA, DON, IMA and BEN, in a  
6 deterministic way. A maximal realistic intestinal concentration of each of these dietary  
7 contaminants has been calculated from the dilution hypothesis of TMDI in the gastro-  
8 intestinal fluid. Maximal realistic intestinal concentrations for experimental use with  
9 intestinal cells have been chosen in the same range of magnitude as calculated ones,  
10 which means 1 ng/ml (2.46 nM) for OTA, 250 ng/ml (840 nM) for DON, 2500 ng/ml  
11 (8400 nM) for IMA and 250 ng/ml (860 nM) for BEN. Details of calculation of the  
12 TMDI and of realistic intestinal concentrations are shown in Table 1 for OTA and DON  
13 and in Table 2 for IMA and BEN. For the assays with H4IIE DR-CALUX<sup>®</sup>, HepG2-Luc  
14 and MMV-Luc cells, as well as with *E. coli* strains, a range of concentrations including  
15 the calculated maximal realistic intestinal concentration was used.

### 17 *Cytotoxicity*

18 Cytotoxicity of the contaminants has been assessed in the various cell lines by the means  
19 of different colorimetric assays evaluating the metabolic activity (considered as a good  
20 approximation of the cell number) during the proliferation phase (MTS and MTT) or the  
21 formation of cell necrosis (LDH release in culture medium) in 16-day post-confluent  
22 cells.

23  
24 In intestinal Caco-2 cells, cytotoxicity of OTA, DON, IMA and BEN was assessed at the  
25 estimated realistic intestinal concentration (see above). After 48h exposure in these  
26 conditions, the MTS assay revealed a significant inhibition of cell proliferation ( $p < 0.005$ )  
27 by DON of approximately 40% in respect to untreated cells (not shown). No significant  
28 effect was observed for the other contaminants in comparison with respective vehicle  
29 controls (not shown). On the other hand, after 6 or 24h exposure of 16-day post-confluent  
30 Caco-2 cells to OTA, DON, IMA or BEN at realistic intestinal concentration, LDH  
31 activity in culture media of treated cells was not higher than in control untreated cells (not

1 shown). In all cases, vehicle controls had no effect on cell proliferation or LDH release in  
2 culture medium (<1% of positive control). No visual alterations were observed under  
3 phase contrast microscope.

4  
5 Cell viability was also assessed by the mean of the MTT assay, with the stable reporter  
6 rat and human hepatoma cell lines (H4IIE DR-CALUX<sup>®</sup> and HepG2-Luc) and the stable  
7 reporter human breast cancer cell line (MMV-Luc) exposed during 24h to a range of  
8 concentration of OTA, DON, IMA or BEN, as described in materials and methods. The  
9 percentage of reduction of cell viability was determined in respect to untreated cells. In  
10 all cases, vehicle controls had not effect on cell viability. Results indicate that none of the  
11 contaminants were cytotoxic for the tested cell lines at the reference calculated realistic  
12 intestinal concentrations considered for this study (see above) (not shown). However,  
13 significant reductions of cell viability of approximately 20% or 30% occurred  
14 respectively in HepG2-Luc cells at 400 ng OTA/ml and in H4IIE DR-CALUX<sup>®</sup> cells at  
15 40 µg OTA/ml, while the MMV-Luc cell line was not significantly affected, whatever the  
16 OTA concentration used. A significant reduction of cell viability was observed after  
17 exposure to concentrations of DON of 400 ng/ml and 2 µg/ml in the three reporter cell  
18 lines. This decrease was approximately of 34%, 20% and 28% at 400 ng/ml and of 52%,  
19 18% and 40% at 2 µg/ml in, respectively, H4IIE DR-CALUX<sup>®</sup>, HepG2-Luc and MMV-  
20 Luc cells. Significant effects of IMA on cell viability were recorded at 20 µg/ml for  
21 H4IIE DR-CALUX<sup>®</sup> cells (±24% of decrease of viability), and at 40 µg/ml for HepG2-  
22 Luc cells (±40% of decrease of viability). IMA was not cytotoxic in the MMV-Luc cell  
23 line, in the range of concentrations tested. BEN reduced cell viability only at the highest  
24 tested dose (40 µg/ml) (± 35% in H4IIE DR-CALUX<sup>®</sup> and HepG2-Luc cells and ±78% in  
25 MMV-Luc cells).

### 26 27 ***Bacterial stress gene profiling assay***

28 Prokaryotic gene expression profiles were obtained following 90 min exposure of the  
29 different transgenic *E. coli* strains used to different concentrations of OTA (1.6 – 100  
30 µg/ml), DON (0.031 – 2 µg/ml), IMA (0.4 – 13 µg/ml) or BEN (0.78 – 50 µg/ml). When  
31 the lowest concentration of the tested range was higher than the calculated realistic

1 intestinal concentration and if no significant effect was observed at this lowest  
2 concentration, effects at lower concentrations were not investigated.

3 In the case of OTA, significant inductions of genes related to membrane (*MicF*) and  
4 DNA (*UmuDC*, *Ada* and *SfiA*) damages were obtained, but only starting from 3.13 µg/ml,  
5 which represents more than 10000 times the calculated realistic intestinal concentration.  
6 At concentrations of 12.5, 25 and 50 µg/ml clear dose-responses were observed for  
7 different stress genes related to oxidative damage (*KatG*, *Zwf*, *Soi28* and *Nfo*), membrane  
8 damage and general cell stress (*MicF*, *OsmY*, *UspA* and *ClpB*) and DNA damage  
9 (*UmuDC*, *Ada*, *DinD* and *SfiA*) (not shown). In the same way, no significant inductions of  
10 stress genes were found after exposure of the transgenic *E. coli* strains to BEN at the  
11 lowest tested concentration (0.78 µg/ml), representing more than 3 times the calculated  
12 realistic intestinal concentration. At higher concentration (12.5 µg/ml), significant  
13 inductions were revealed for endpoints related to membrane damage (*MicF*) and general  
14 cell lesions induced by protein perturbation (*ClpB*) (not shown). Regarding DON, only  
15 one marker for DNA damage (*DinD*) was induced upon exposure to the calculated  
16 realistic intestinal concentration of 250 ng/ml (Figure 2A). Furthermore, at a  
17 concentration ten times higher, significant induction for *RecA*, which is part of the SOS  
18 response, was found. *E. coli* strains were also sensitive to IMA. At 2500 ng/ml, the  
19 realistic intestinal concentration, statistically significant inductions were found for  
20 markers related to protein perturbation, membrane integrity and osmotic stress (*MicF* and  
21 *ClpB*) and oxidative stress (*Zwf*, *Soi28* and *SfiA*) (Figure 2B).

### 22 23 ***Modulation of intestinal cytochrome P-450 biotransformation enzyme activity***

#### 24 Induction of CYP1A1 activity

25 The effect of OTA, DON, IMA and BEN at calculated realistic intestinal concentration  
26 (see above) on CYP1A1 activity in Caco-2 cells after 6 and 24h exposure is illustrated in  
27 Figure 3. While OTA, DON and BEN had no effect, IMA revealed to be a significant  
28 time-dependent CYP1A1 inducer ( $p < 0.005$ ), with a stronger induction after 24 than 6h  
29 exposure. Vehicle controls had no inductive effect on CYP1A1 activity in comparison  
30 with untreated cells (not shown).

#### 31 Inhibition of CYP3A4 activity

1 The inhibitory effect of OTA, DON, IMA and BEN on CYP3A4 has been tested in Caco-  
2 2 cells previously treated during 14 days with 1,25-vitD<sub>3</sub>, in order to express functional  
3 CYP3A4 activity (Schmiedlin-Ren *et al.*, 1997). The cells were then exposed during 3h  
4 simultaneously to 500 µM of testosterone and to OTA, DON, IMA or BEN at the  
5 estimated realistic intestinal concentration (see above). The quantity of 6β-(OH)-  
6 testosterone secreted was then measured in the cell medium. The test revealed a strong  
7 inhibition of CYP3A4 activity by IMA ( $p < 0.05$ ;  $\pm 60\%$  in respect to negative control  
8 consisting in untreated but pre-induced cells), as strong as by ketoconazole (positive  
9 control). The activity levels achieved were down to the value measured with non-induced  
10 cells, representing the basal CYP3A4 activity without preliminary induction with 1,25-  
11 vitD<sub>3</sub> (Figure 4). Other contaminants (OTA, DON and BEN) as well as vehicle controls  
12 had no significant inhibitory effect on CYP3A4 activity.

13

#### 14 ***Human and rat aryl hydrocarbon receptor (AhR) agonistic/antagonistic activities***

15 Exposure of dioxin-responsive cells (H4IIE DR-CALUX<sup>®</sup> and HepG2-Luc) to the  
16 reference ligand (TCDD) during 24h resulted in a dose-response relationship (not  
17 shown). The median effective concentrations (EC<sub>50</sub>) were 0.004 and 0.5 ng TCDD/ml in  
18 H4IIE DR-CALUX<sup>®</sup> and HepG2-Luc cells, respectively, showing the higher sensitivity  
19 to TCDD of rat hepatoma cells compared to human hepatoma cells. Time exposure (6h vs  
20 24h) did not change the cell sensitivity to TCDD (not shown).

21 H4IIE DR-CALUX<sup>®</sup> or HepG2-Luc stable reporter cells were exposed to a range of  
22 concentrations of OTA, DON, IMA or BEN during 6 and 24h. No luciferase activity,  
23 resulting from the activation of the AhR, was observed.

24

25 In antagonistic assays, H4IIE DR-CALUX<sup>®</sup> or HepG2-Luc cells were exposed during  
26 24h simultaneously to TCDD and a range of concentrations of OTA, DON, IMA or BEN.  
27 No AhR antagonistic activity was observed for none of the tested chemicals at the  
28 reference concentrations considered in this paper, which are the realistic intestinal  
29 concentrations, as well as at all non cytotoxic concentrations tested.

30 In all cases, vehicle controls had no effect on AhR activation.

31

### 1 **Human estrogen receptor (hER) agonistic/antagonistic activities**

2 Exposure of MMV-Luc cells to the reference ligand E2 during 24h resulted in a dose-  
3 dependent relationship (Figure 5), with an EC<sub>50</sub> of 0.003 ng/ml. Fulvestrant (ICI 182,  
4 780) is a high affinity ER antagonist (Osborne *et al.*, 2004). As a control, the antagonistic  
5 activity was assayed in cells simultaneously exposed to E2 (1.4 ng/ml) and fulvestrant  
6 (60.7 ng/ml). A 93% inhibition of the E2-related induction was observed, confirming the  
7 antagonistic activity of fulvestrant in MMV-Luc cells, while no cytotoxic activity was  
8 evidenced using the MTT assay (not shown).

9  
10 OTA, DON and IMA showed no hER agonistic activity in MMV-Luc cells exposed  
11 during 24h to the range of concentrations. On the contrary, we obtained a dose-response  
12 relationship when cells were exposed to BEN with an EC<sub>50</sub> of 475 ng/ml (Figure 5). The  
13 REP of BEN expressed as the ratio between the EC<sub>50</sub> of E2 and BEN was therefore 6.3  
14 10<sup>-6</sup>. At the reference concentration considered in this paper, which is the realistic  
15 intestinal concentration (250 ng/ml), the *in vitro* estrogenic activity of BEN was  
16 equivalent to the effect produced by the EC<sub>50</sub> (0.003 ng/ml) of E2.

17 In antagonistic assays, MMV-Luc cells were exposed during 24h simultaneously to E2  
18 and a range of concentrations of OTA, DON, IMA or BEN. In these conditions, none of  
19 the tested contaminants were able to decrease the relative response induced by E2 at non  
20 cytotoxic concentrations.

21 In all cases, vehicle controls had no effect on hER activation.

### 22 23 **Discussion**

24 In the present paper, different cellular responses to four selected dietary chemical  
25 contaminants (OTA, DON, IMA and BEN) were assessed at realistic human exposure. A  
26 comparison of the results obtained with those from the literature has been performed in  
27 order to validate and propose this methodology as a suitable toolbox to take a first *in vitro*  
28 'fingerprint' of less studied dietary toxicants for risk assessment studies.

29 Since in risk assessment studies hazard characterization of dietary chemicals should  
30 ideally be performed at exposure levels representative of 'normal consumers', we have  
31 first proposed a methodology allowing the estimation of maximal realistic concentrations



1 of dietary contaminants occurring in the gastro-intestinal tract by using a dilution  
2 hypothesis of the ingested daily intake in the intestinal lumen. The deterministic approach  
3 followed allowed us to easily mimic intestinal chronic exposure with *in vitro* systems to a  
4 broad range of contaminants, including those for which no ADI/TDI has been established  
5 and no databases necessary in a probabilistic approach are available. The theoretical  
6 maximum daily intake (TMDI) has been obtained by multiplication of European maximal  
7 authorized level of contaminants in foodstuffs (contamination data) with average adult  
8 daily consumption data of these foodstuffs from GEMS/FOOD Regional Diets, which are  
9 based on national Food Balance Sheets. Data from GEMS/FOOD Regional diets greatly  
10 underestimate short-term high percentile intakes. They are used in chronic exposure  
11 assessment by the Joint Meeting on Pesticide Residues (JMPR) and the Joint FAO/WHO  
12 Expert Committee on Food Additives (JECFA), according to internationally accepted  
13 methodologies (WHO, 1997), in order to assess long-term risks at the international level.  
14 This method permits thus a valid comparison of TMDI with the 'Acceptable Daily  
15 Intake' (ADI, for pesticides) or 'Tolerable Daily Intake' (TDI, for other contaminants),  
16 which are based on long-term food consumption habits.

17  
18 This very simple deterministic method, called 'by point estimates', has the particularity  
19 not to be associated with any measurement of confidence and to slightly over-estimate  
20 actual exposure, since it assumes that all individuals consume the same quantities of  
21 specified foodstuff(s) and that the contaminant is always present in the foodstuff(s) at a  
22 high level (Kroes *et al.*, 2002). On the other hand, as compared to the probabilistic  
23 approach, which is based on distributions rather than on single-point values, it requests  
24 less extensive databases and offers more easily interpretable results (Claeys *et al.*, 2008).  
25 It is therefore commonly used as a first step in exposure assessment and most appropriate  
26 for screening purposes (Verger, 2006), in accordance with our study. The probabilistic  
27 method is indeed very useful when the first deterministic estimation is at or above  
28 ADI/TDI, since it allows to refine the assessment and to better evaluate if the chemical  
29 exposure is a public health issue. Whatever the approach used, it should be kept in mind  
30 that the quantity of contaminant residues effectively occurring in food can be lower than  
31 in raw materials after processing and/or storage (Kroes *et al.*, 2002).

1 In the present study, the main sources of ingestion of OTA, DON, IMA and BEN for  
2 which European M(R)Ls in foodstuffs exist, have been taken into account in the  
3 calculation of TMDI. For OTA, European MLs in cereal-based products, contributing to  
4 50-70% of the dietary intake (Petzinger and Ziegler, 2000), dried vine fruit, coffee and  
5 wine have been considered, while for DON, European MLs in cereal-based products were  
6 the only ones to be used. The exposure assessment has resulted in a daily intake for OTA  
7 and DON of respectively 13.05 ng/kg bw/day, representing 76% of TDI (considering that  
8 TWI is equally spread over the week) and 2.37 µg/kg bw/day, or 237.7% of TDI. The fact  
9 that TMDI exceeds TDI may be explained by the crude over-estimation induced by the  
10 method. It is a preliminary indication that a potential risk should be taken into account  
11 and that a more precise exposure assessment should be performed, for example with the  
12 probabilistic method. By comparison, the European Scientific Cooperation (SCOOP)  
13 tasks on mycotoxins give estimated daily intakes of 0.1-3.5 ng/kg bw/day for OTA and  
14 0.078-0.48 µg/kg bw/day for DON, for participating member states displaying the lowest  
15 and highest mean exposure (EC, 2002; EC, 2003). For OTA, other studies report dietary  
16 exposure of 2-3 ng/kg bw/day for the average adult consumer (EFSA, 2006) or 0.7-4.6  
17 ng/kg bw/day for average consumers and 6-8 ng/kg bw/day for high consumers (SCF,  
18 1998). In 2002, JECFA published a report where a probabilistic approach with French  
19 data resulted in an intake of 92 ng OTA/kg bw/week (or 13.14 ng/kg bw/day) for  
20 consumers of cereals at the 95<sup>th</sup> percentile. Regarding DON, an estimation of the dietary  
21 intake ranged from 0.77 µg/kg bw/day with the African diet to 2.4 µg/kg bw/day with the  
22 Middle Eastern diet (WHO, 2001).

23  
24 The oral exposure of human to IMA and BEN through consumption of contaminated  
25 fruits and vegetables has been assessed in the present study to respectively 17.47 µg/kg  
26 bw/day and 3.34 µg/kg bw/day and represents respectively 58.2% and 3.3% of their ADI.  
27 In a study based on a Belgian pesticide monitoring program, the authors calculated an  
28 exposure for the 97.5<sup>th</sup> percentile consumer reaching ±25% and ±2% of ADI for IMA and  
29 BEN, respectively (Claeys *et al.*, 2008). In USA, the exposure to IMA was estimated to  
30 0.05 µg/kg bw/day, in the worst case, by considering residue data in products prepared as  
31 for consumption (Dougherty *et al.*, 2000). In the case of IMA, an overestimation of the

1 actual exposure by our methodology may be caused by the fact that residues are mainly  
2 located in the peel of citrus fruits, often removed before consumption.

3  
4 The dilution model of calculated TMDI in the gastro-intestinal fluid used in the present  
5 work to generate realistic intestinal concentrations does not take into account inter-  
6 individual variations, which can obviously influence the amount of contaminants released  
7 from the food matrix in the gastrointestinal tract during the digestion process and  
8 accessible for intestinal absorption (*i.e.* gastric and intestinal lumen volume, rate of saliva  
9 secretion, rate of pancreatic or biliary secretions, composition of these secretions). In  
10 addition, the approach chosen in the present study does not at all allow estimating the  
11 concentrations of the contaminants in the body fluids. The concentration of the  
12 contaminant in the blood depends on individual-specific factors including the proportion  
13 of contaminant absorbed by intestinal cells and released in the bloodstream, the first-pass  
14 metabolism in intestinal and liver cells, the excretion back towards the lumen, the half-  
15 life and the degree of serum binding (mainly albumin), which can reach more than 99%  
16 in the case of OTA (Ringot *et al.*, 2006). The literature gives a few data for the presence  
17 of contaminants in biological fluids. Depending on the study, the occurrence of OTA in  
18 human blood ranged from 0.2 to 0.88 ng/ml (Studer-Rohr *et al.*, 2000), from 0.17 to 0.56  
19 ng/ml (EFSA, 2006) or from 0.18 to 1.19 ng/ml (EC, 2002), concentrations very close to  
20 the calculated realistic intestinal concentration of the present study (1 ng/ml). In humans,  
21 the concentration of DON in blood is poorly documented, while in urines, some authors  
22 found mean levels of 37 ng/ml and 12 ng/ml, respectively in high and low-risk areas in  
23 China (Meky *et al.*, 2003). To our knowledge, concentrations of IMA and BEN in human  
24 body fluids have never been measured.

25  
26 As a prerequisite to *in vitro* assays, the cytotoxicity of OTA, DON, IMA and BEN was  
27 assessed and further experiments were performed at non-cytotoxic concentrations. DON  
28 revealed to be toxic in almost all cell types, even at realistic intestinal concentration (250  
29 ng/ml) with proliferating Caco-2 cells. The inhibition of cell proliferation by DON in  
30 intestinal cell lines was already shown in similar conditions of exposure (Maresca *et al.*,  
31 2002; Kouadio *et al.*, 2005; Bony *et al.*, 2006; Sergent *et al.*, 2006; Van De Walle *et al.*,

1 2008), whereas in the HepG2 cell line, an IC<sub>50</sub> of 8.4 µg/ml has been calculated (Cetin  
2 and Bullerman, 2005), suggesting that these cells are less sensitive to DON than Caco-2  
3 cells. In the present study, a realistic exposure of 16-day post-confluent Caco-2 cells to  
4 DON has not resulted in a marked increase of LDH release, a marker of cell necrosis. On  
5 the contrary, cytotoxicity tests performed with DON in the intestinal cell line HT-29-D4,  
6 showed a very slight but significant increase of LDH release (less than 2.5%) from 10 nM  
7 (2.96 ng/ml), while 100 µM of DON (29 µg/ml) was necessary to strongly increase LDH  
8 release (642% of control value), induce cellular apoptosis and diminish protein content  
9 (Maresca *et al.*, 2002). Intestinal necrosis is indeed a major toxic effect of DON. *In vivo*,  
10 the ingestion of contaminated food with high doses of DON may create lesions of the  
11 mucosa in the gastrointestinal tract, resulting in extensive hemorrhage, vomiting and  
12 gastroenteritis symptoms (Pestka and Smolinski, 2005). The cytotoxicity of DON has  
13 often been attributed to the inhibition of protein synthesis, by impairment of the peptidyl-  
14 transferase on the 60S ribosomal subunit. As a translational inhibitor binding to  
15 ribosomes, DON rapidly activates the double-stranded RNA-activated protein kinase R  
16 (PKR) and the downstream mitogen-activated protein kinase (MAPK) cascades, which  
17 drive apoptosis as well as activation of transcription factors implicated in the  
18 inflammatory pathway (Zhou *et al.*, 2003; Pestka and Smolinski, 2005; Van De Walle *et*  
19 *al.*, 2008). This overall process is called 'ribotoxic stress response'. The impact of DON  
20 on cell viability was furthermore explained by pointing mitochondria and lysosomes as  
21 targets, by showing an inhibition of protein and DNA synthesis, as well as through lipid  
22 peroxidation (Kouadio *et al.*, 2005; Kouadio *et al.*, 2007). Interestingly enough, we  
23 observed that the *E. coli* strain transfected with the promoter *DinD*, responsive to DNA  
24 damage, was induced at calculated realistic intestinal concentration. Furthermore, at a  
25 concentration ten times higher, significant inductions for *RecA*, part of the SOS response,  
26 were found. These results are in accordance with the genotoxic potential attributed to  
27 DON (Rotter *et al.*, 1996; Bony *et al.*, 2006). In addition to information about its mode of  
28 action, this assay suggests that the toxicity of DON towards bacteria at realistic exposure  
29 may imbalance gastro-intestinal populations, which may, on one hand, impair the  
30 metabolism and bioavailability of food compounds and xenobiotics, and, on the other  
31 hand, modify the systemic effects of the gut microbiota.

1 At realistic intestinal concentration, no cytotoxicity of OTA has been highlighted in  
2 intestinal cells. Sensitivity of intestinal cells to OTA toxicity has yet been shown *in vitro*  
3 but at higher concentrations than those used in the present work (Berger *et al.*, 2003;  
4 Creppy *et al.*, 2004). The kidney is generally considered as the first and major organ  
5 affected by OTA. Nevertheless, the IC<sub>50</sub> calculated in intestinal cells are often lower than  
6 in other studies using renal cells, suggesting that the kidney has no specific sensitivity  
7 towards OTA-induced cytotoxicity in comparison with cells of other origin (Creppy *et*  
8 *al.*, 2004; Kamp *et al.*, 2005), even if nephropathy is the main *in vivo* reported effect. The  
9 unchanged levels of LDH activity between control and OTA-treated intestinal cells,  
10 confirmed by Berger *et al.* (2003), suggest that OTA does not induce cell necrosis in the  
11 intestine at realistic intestinal concentration. Kamp *et al.* (2005) observed OTA-  
12 dependent oxidative DNA damage, suggesting that oxidative stress plays an important  
13 role in OTA-derived toxicity/carcinogenicity in the kidney, whereas another hypothesis  
14 for OTA carcinogenicity suggests covalent DNA adduct formation (for a review, see  
15 Pfohl-Leszkowicz and Manderville (2007)). In the present study, bacterial stress genes  
16 related to membrane, DNA and oxidative damage are significantly induced at high  
17 concentration. The inductions of *SfiA* and *Nfo* more specifically suggest oxidative DNA  
18 damage (Dardenne *et al.*, 2008).

19  
20 IMA revealed no cytotoxicity in eukaryotic cell lines in a realistic range of  
21 concentrations. Little information is available regarding toxic effects of IMA. Different  
22 studies have established oral LD<sub>50</sub> in rat ranging from 227 to 664 mg/kg bw. It seems that  
23 the liver is the primary target for IMA in experimental animals (EFSA, 2007) and a time-  
24 dependent cell death, accompanied by the depletion of intracellular ATP, GSH and  
25 protein thiols, was observed in isolated rat hepatocytes by Nakagawa and Moore (1995).  
26 These authors have suggested that cytotoxicity caused by IMA might be related to lipid  
27 peroxidation, due to an oxidative stress, as well as to the depletion of ATP via  
28 impairment of the mitochondrial function. In the present study, we have shown in the  
29 prokaryotic gene expression profile of IMA that significant inductions of genes related to  
30 cellular and osmotic stress, as well as to oxidative stress, occur at realistic gastro-  
31 intestinal exposure. These results are in accordance with the hypothesis that IMA

1 provokes an oxidative stress, as suggested by Nakagawa and Moore (1995). As a  
2 consequence of the toxicity of a realistic dose of IMA on gastro-intestinal bacteria, an  
3 imbalance of bacterial populations and/or a change in metabolism in the lumen may  
4 occur.

5  
6 The fungicide BEN commonly induces hepatotoxicity (Igbedioh and Akinyele, 1992;  
7 McCarroll *et al.*, 2002). However, in the present study, none of the tested cell lines  
8 showed reduced viability in a biological realistic range of concentrations. In a study  
9 performed with the HepG2 cell line, Dierickx (1999) observed a reduced cell viability at  
10 a non-physiological concentration of BEN, similar to the concentration showing reduced  
11 viability of HepG2-Luc cells in the present work. He attributed the observed effects to the  
12 butylcarbamoyl group of BEN. Interestingly enough, an *in vivo* study (Banks and  
13 Soliman, 1997) has shown that a 7-day treatment of rats significantly increased lipid  
14 peroxidation and depleted liver GSH levels, suggesting that the hepatotoxicity induced by  
15 BEN or its metabolites is due to an oxidative stress of cellular membranes. In the present  
16 work, even though no response was recorded in the bacterial gene expression assay at  
17 realistic concentration, significant inductions were observed at higher concentrations for  
18 membrane damage-related endpoints, supporting the idea that lipid peroxidation may be  
19 one of the modes of action of BEN.

20  
21 Once absorbed from the lumen into intestinal cells, chemicals may undergo first-pass  
22 metabolism. CYPs are major actors of these biotransformations. The study of the effects  
23 of dietary chemicals on CYP expression and/or activity is of primary importance in the  
24 comprehension of mechanisms of adverse and beneficial interactions in cells (Sergent *et al.*,  
25 2008). Since CYPs have often broad substrate specificities, their induction by one  
26 chemical may result in enhanced metabolism of other chemicals or drugs, thereby,  
27 altering their bioavailability, pharmaco- or toxicokinetic properties as well as pharmaco-  
28 or toxicodynamic effects (Ma, 2001).

29  
30 As in a previous publication from our group (Sergent *et al.*, 2009), we have again  
31 highlighted that IMA, which is commonly recovered in consumed fruits and vegetables,

1 induced CYP1A1 activity at realistic exposure in intestinal Caco-2 cells. However, we  
2 have not shown an AhR-mediated agonistic activity of IMA with the reporter gene assay.  
3 Our results are in accordance with those of a screening study on 200 pesticides with  
4 mouse hepatoma (Hepa1c1c7) cells stably transfected to obtain dioxin-responsive  
5 luciferase reporter cells (Takeuchi *et al.*, 2008). This suggests that the classical AhR  
6 pathway does not mediate the induction of CYP1A1 activity by IMA. An alternative  
7 CYP1A1 regulation pathway, involving PPAR- $\alpha$ , another ligand-activated transcription  
8 factor, and two PPAR response elements (PPRE) sites located within the CYP1A1  
9 promoter gene, has been proposed in Caco-2 cells (Sérée *et al.*, 2004; Fallone *et al.*,  
10 2005). Since it was previously observed that IMA is able to induce PPAR- $\alpha$ -mediated  
11 transcriptional activity in reporter cells (Takeuchi *et al.*, 2006), the PPAR- $\alpha$ -mediated  
12 CYP1A1 regulation pathway might be considered as potentially responsible for the  
13 observed induction of CYP1A1 activity by IMA in Caco-2 cells. The induction of  
14 CYP1A1 activity by IMA has been previously demonstrated in the small intestine and  
15 liver of rats as well as in Caco-2 cells (Muto *et al.*, 1997; Sergent *et al.*, 2009). However,  
16 Sergent *et al.* (2009) have suggested that a post-transcriptional pathway, acting on the  
17 protein rather than on the gene expression, is involved in the increase of CYP1A1 activity  
18 following exposure of Caco-2 cells to IMA.

19  
20 OTA, DON and BEN had no inductive effect on CYP1A1 activity at realistic intestinal  
21 concentration. In accordance with these results, no AhR-mediated agonistic activity was  
22 observed in the present study after exposure of reporter cells to different concentrations  
23 of these contaminants. In the same trend, Takeuchi *et al.* (2008), who screened 200  
24 pesticides with mouse hepatoma (Hepa1c1c7) cells stably transfected to obtain dioxin-  
25 responsive luciferase reporter cells, did not observe any AhR agonistic activity after  
26 exposure to BEN. However, induction of CYP1A1 activity and expression by BEN and  
27 its derivative carbendazim have been demonstrated in HepG2 cells and rabbit  
28 hepatocytes, but at concentrations much higher than those used in the present study (Rey-  
29 Grobellet *et al.*, 1996; Dierickx, 1999).

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1 It is well known that CYP1A1 is implicated in the conversion of pro-carcinogens from  
2 the PAH family to reactive cytotoxic metabolites, such as carcinogenic DNA-binding  
3 species (Schwarz *et al.*, 2001; Shimada *et al.*, 2001). An induction of CYP1A1 activity by  
4 another compound, such as IMA in the present case, might further provoke either pro-  
5 carcinogen activation or detoxification by hydroxylation. Toxicity *vs* protection resulting  
6 from CYP1A1 induction appears to depend on the cellular context, such as carcinoma  
7 cells *vs* intact animal (Uno *et al.*, 2004), to tissue- or cell type- specificities as well as to  
8 the involvement of phase II metabolism (Nebert *et al.*, 2004). This comment emphasizes  
9 the extreme care that has to be taken in the extrapolation of *in vitro* results to the *in vivo*  
10 situation. Furthermore, it is difficult to conclude about consequences of CYP1A1  
11 induction since other effects may arise in parallel, such as inhibition of TCDD-induced  
12 CYP1A1 activity by IMA in Caco-2 cells, suggesting cytoprotective effects towards pro-  
13 carcinogen activation (Sergent *et al.*, 2009).

14  
15 In addition to xenobiotic metabolism, the CYP1A1 isoform is also involved in the  
16 transformation of endobiotic compounds, such as *at*RA, the active metabolite of retinol  
17 (vitamin A) (Lampen *et al.*, 2000) and E2, which is known to act both as a promoter of  
18 cell proliferation and as a pro-carcinogen (Tsuchiya *et al.*, 2005; Parl *et al.*, 2009). The  
19 induction of CYP1A1 activity may thus lead in the disruption of *at*RA biological  
20 activities, as well as in the resistance at the target site when *at*RA is used clinically. It  
21 may also increase the metabolism of E2 to weak estrogenic (Oh and Chung, 2006), but  
22 potentially toxic metabolites (Tsuchiya *et al.*, 2005; Parl *et al.*, 2009). Therefore,  
23 intestinal CYP1A1 inducers, such as IMA, may influence the *at*RA and E2 small  
24 intestinal metabolism and action.

25  
26 Results from the present work confirm previously published material (Sergent *et al.*,  
27 2009) that shows a strong inhibition of CYP3A4 activity by a realistic concentration of  
28 IMA in 1,25-vitD<sub>3</sub>-induced Caco-2 cells, but at lower concentration than ketoconazole, a  
29 known CYP3A4 inhibitor in this cell line (Gibbs *et al.*, 2000; Hou *et al.*, 2007). The  
30 inhibition of the CYP3A4 activity but not gene transcription by IMA has been previously  
31 observed in Caco-2 cells by Sergent *et al.* (2009). On the other hand, the induction of



1 CYP3A4 activity and expression, as well as the activation of the human pregnane X  
2 receptor (hPXR), by IMA have been previously demonstrated (Muto *et al.*, 1997;  
3 Lemaire *et al.*, 2006b). A broad range of drugs, carcinogens, but also endogenous steroids  
4 (testosterone, progesterone, androstenedione, and cortisol) or E2 are substrates of  
5 CYP3A4 (Guengerich, 1999; Goodwin *et al.*, 2002) and thus good candidates for  
6 interactions at the intestinal level. Since more than the half of the existing drugs is  
7 metabolized by CYP3A4, interactions with drugs constitute the main issue when foreign  
8 compounds modulate CYP3A4 activity. For example, an increase of bioavailability and  
9 plasma concentration of felodipine or of the pro-drug terfenadine, due to the diminution  
10 of oxidation by CYP3A4 inhibitors such as grapefruit juice or ketoconazole, has been  
11 associated with detrimental effects. On the other hand, it has been demonstrated that the  
12 CYP3A4 inhibitor ketoconazole increases oral bioavailability of concurrently  
13 administered cyclosporine and reduces the dosage required (Guengerich, 1997; Dresser *et al.*,  
14 2000). Therefore, the co-occurrence at the intestinal level of IMA and a CYP3A4-  
15 substrate drug may alter its efficiency, either adversely or positively, by pre-systemic  
16 metabolism. In addition, the inhibition of CYP3A4 activity by IMA may have an impact  
17 on the metabolism of food contaminants, since CYP3A4 activates some PAH  
18 dihydrodiols (Shimada *et al.*, 2001), as well as pro-carcinogens, such as aflatoxin B1  
19 (AFB1) (Shimada and Guengerich, 1989; Guengerich and Shimada, 1998), but at  
20 relatively high substrate concentration (Gallagher *et al.*, 1994).

21  
22 The ability of BEN to display hER agonistic activity, even at realistic gastro-intestinal  
23 concentration, has been demonstrated in the present paper. The activation of the  
24 transcription of the firefly luciferase reporter gene probably occurs after the binding of  
25 the dimer complex 'ligand (BEN) - receptor (ER)' to the ERE elements present in the  
26 vitellogenin promoter of the MMV-Luc cell line. An agonistic effect of BEN towards  
27 ER $\alpha$  has indeed been reported previously in reporter HeLa cells (Lemaire *et al.*, 2006a).  
28 Furthermore, BEN was previously shown to bind the recombinant hER $\alpha$  in a binding  
29 assay (Scippo *et al.*, 2004). The estrogenicity of BEN and its degradation product  
30 carbendazim was also shown to act via the induction of the aromatase CYP19 activity,  
31 catalyzing the conversion of androgens to estrogens, in a human ovarian granulosa-like

1 tumor cell line (Morinaga *et al.*, 2004). Therefore, it is likely that BEN acts as an  
2 estrogenic compound by affecting both the action of estrogens, through binding to ER $\alpha$   
3 and activation of ERE downstream genes, and the synthesis of estrogens, by increasing  
4 estrogens level. We therefore suspect that BEN acts as an endocrine disruptor, which may  
5 lead to estrogen-mediated pathologies such as reproductive disorders.

6  
7 The overall toolbox approach proposed in this paper and including four types of *in vitro*  
8 assays revealed to be interesting in assessing chemical risks at the intestinal level upon  
9 dietary consumption. In a first assay, genetically modified *E. coli* strains responding to  
10 different types of stress permitted to assess toxicity on bacteria present in the human gut,  
11 potentially leading to an imbalance in the populations and a modification of their activity  
12 and in turn impacting the digestive function, the absorption and more generally some of  
13 the various effects of the microbiota on the human health. The second *in vitro* assay  
14 performed with intestinal Caco-2 cells allowed to give a quick insight into the modulation  
15 of the activity of CYP1A1 and CYP3A4 in small intestinal cells. The third and fourth *in*  
16 *vitro* assays focused on the AhR and ER, respectively. Whereas the AhR is undoubtedly  
17 present in human enterocytes (Fallone *et al.*, 2004), controversy remains regarding the  
18 presence of the ER. Nevertheless, ER has been identified and localized in the mouse  
19 small intestine (Kawano *et al.*, 2004) and in the human colon (Singh *et al.*, 1998).  
20 Furthermore, its expression has been associated with a lower incidence of small intestinal  
21 tumorigenesis in mice (Giroux *et al.*, 2008) and estrogen exposure in women results in a  
22 reduction in colon cancer risk (Grodstein *et al.*, 1999). In addition, recently, the decrease  
23 of permeability of the intestinal barrier by ER agonists, including E2, has been  
24 demonstrated both in rodents and in intestinal cell lines, such as Caco-2 (Braniste *et al.*,  
25 2009; Looijer-van Langen *et al.*, 2011). AhR- or ER-mediated (ant)agonism by dietary  
26 chemical compounds reaching the intestinal barrier at high concentration might therefore  
27 have important consequences for human health.

28 The proposed toolbox is a first initiative to associate different complementary, friendly-  
29 to-use and relevant tools. It may be further improved in the future with additional  
30 approaches such as the inclusion of an *in vitro* simulated digestion of dietary chemicals

1 followed by absorption studies on intestinal cells, of inhibitory assays on CYP1A1 or  
2 through the use of intestinal luciferase reporter cells responding to AhR and ER ligands.

## 3 4 **Conclusions**

5 In the present work, we have proposed a package of *in vitro* experimental tools, which  
6 responded to chemical stresses and proved to be efficient in assessing some relevant  
7 dietary toxicological effects, even at realistic contaminant exposure in humans, as shown  
8 by the comparison of the results obtained with four well-known contaminants with those  
9 from the literature. Due to its high contribution in first-pass metabolism, as well as in the  
10 bioavailability of dietary compounds, the intestinal compartment was the major focus of  
11 the present study. Therefore, a method of easy evaluation of realistic concentrations of  
12 dietary contaminants occurring in the human gastro-intestinal tract was proposed. This  
13 method can be applied to a large range of dietary chemicals for which limited data exist  
14 and is relevant in the risk assessment process.

15  
16 This overall approach is not time-consuming and may be applied to high throughput  
17 screening of high numbers of samples. It allows to take a first ‘fingerprint’ of tested  
18 dietary contaminants at realistic exposure, which may be useful for further risk  
19 assessment studies and for limiting *in vivo* testing. The proposed experimental toolbox,  
20 which gathers diverse yet existing test systems, offers an additional dimension in  
21 comparison with the *in vitro* assays taken separately. In the scope of emerging risk  
22 detection, this tool is very promising due to the diversity and complementarities of  
23 endpoints measured and the biological relevance of the concentrations selected for the  
24 experimental studies.

## 25 26 **Acknowledgments**

27 This study was funded by the Belgian Federal Public Service of Health, Food Chain,  
28 Safety and Environment (contract RT 05/07 EMRISK).

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3 **Figure 1: Chemical structure of food contaminants investigated in the present**  
4 **study: ochratoxin A (OTA), deoxynivalenol (DON), imazalil (IMA) and benomyl**  
5 **(BEN).**  
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10 **Figure 2: Prokaryotic gene expression profile after exposure of *E. Coli* strains to a**  
11 **concentration range of (A) DON (0.031 – 2 µg/ml) and (B) IMA (0.4 – 13 µg/ml).**

12 Values are means of three independent experiments performed in triplicates and are  
13 expressed as fold inductions, calculated with formulas as described in materials and  
14 methods. An arrow indicates the calculated realistic intestinal concentration. The  
15 different genes are responsive to: *katG*: oxidative stress, *micF*: membrane integrity and  
16 osmotic stress, *osmY*: osmotic stress, *uspA*: growth arrest, *recA*: SOS response, *zwf*:  
17 oxidative stress, *clpB*: protein perturbation, *umuDC*: radiation and/or chemically induced  
18 DNA damage, *merR*: heavy metals, *ada*: DNA damage (mainly methyl adducts), *dinD*:  
19 DNA damage, *soi28*: superoxide radical generating agents, *nfo*: single- and double-  
20 stranded DNA breaks and oxidative DNA damage, *SfiA*: SOS response to DNA damage.  
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31 **Figure 3: Modulation of CYP1A1 activity by OTA, DON, IMA and BEN in Caco-2**  
32 **cells.** Sixteen-day post-confluent cells seeded in 48-well plates ( $3.10^4$  cells/cm<sup>2</sup>) were  
33 exposed to OTA (1 ng/ml), DON (250 ng/ml), IMA (2500 ng/ml) or BEN (250 ng/ml) for  
34 6 or 24h. Positive controls consisted in cells treated with B[a]P (250 ng/ml) or TCDD  
35 (0.3 ng/ml) and negative control consisted in untreated cells. CYP1A1 activity was  
36 assessed through the measurement of the fluorescence produced by the conversion of 5  
37 µM 7-ethoxyresorufin to resorufin. Results are expressed in pmol resorufin  
38 produced/min/mg protein. Values are means ± S.E.M. of three independent experiments  
39 performed in triplicates (n=9). \*\*\*  $P < 0.005$  as compared to respective vehicle controls.  
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48 **Figure 4: Modulation of CYP3A4 activity by OTA, DON, IMA and BEN in Caco-2**  
49 **cells.** Cells were seeded in 12-well plates ( $4.10^4$  cells/cm<sup>2</sup>) and treated during 14 days  
50 from confluence with 0.5 µM 1,25-vitD<sub>3</sub>, in order to induce CYP3A4 activity. Cells were  
51 then exposed for 3h simultaneously to testosterone (500 µM) and to OTA (1 ng/ml),  
52 DON (250 ng/ml), IMA (2500 ng/ml), BEN (250 ng/ml) or ketoconazole (positive  
53 control) (26.6 µg/ml). The CYP3A4 activity of the cells was then assessed through the  
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3 HPLC determination of 6 $\beta$ -(OH)-testosterone in the cell medium. Non-induced cells  
4 represent the basal CYP3A4 activity without preliminary inductive treatment with 1,25-  
5 vitD<sub>3</sub>. Results are expressed in pmol 6 $\beta$ -(OH)-testosterone produced/min/mg protein and  
6 illustrated in % of negative control (5.13 $\pm$ 0.37 pmol/min/mg protein), consisting in  
7 untreated but pre-induced cells. Values are means  $\pm$  S.E.M. of three independent  
8 experiments performed in triplicates (n=9). \**P* < 0.05, \*\*\**P* < 0.005 as compared to  
9 respective vehicle controls.  
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17 **Figure 5: Dose-response curves of E2 and BEN in MMV-Luc cells.** Exposure time  
18 was 24h. Results are expressed as means of the relative response (%)  $\pm$  S.E.M. (100%  
19 with highest tested concentration of E2), of two independent experiments performed in  
20 triplicates (n=6), in function of the ligand concentration (ng/ml).  
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**Table 1: Theoretical maximum daily intake (TMDI) and realistic intestinal concentration (C) of OTA and DON, estimated by a deterministic approach.**

European maximal legal limits (MLs) in the main consumed foodstuffs specified in the Commission Regulation (EC) N°1881/2006 of 19 December 2006 and human European consumption data from GEMS/FOOD Regional Diets (WHO, 2003) ( $Q_{Ei}$ ) were used (TWI, tolerable weekly intake; TDI, tolerable daily intake; bw, body weight).

	MLs ( $\mu\text{g}/\text{kg}$ )	Consumption data		TMDI ( $\text{ng}/\text{person}/\text{day}$ )
		$Q_{Ei}$ ( $\text{g}/\text{person}/\text{day}$ )	GEMS code	
<b>OTA</b>				
TWI <sup>1</sup> : 120 $\text{ng}/\text{kg}$ bw/week				
Cereal-based products	3	221.9	GC 80	665.7
Dried wine fruits	10	2.3	DF 269	23
Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5	5.8	SB 716ro	29
Wine	2	97.8	DF 269w	195.6
Total ( $\text{ng}/\text{person}/\text{day}$ )				913.3
Total ( $\text{ng}/\text{kg}$ bw/day) <sup>2</sup>				13.05
% TDI <sup>3</sup>				76 %
C ( $\text{ng}/\text{ml}$ )				0.913
<b>C<sub>used</sub> (<math>\text{ng}/\text{ml}</math>)</b>				<b>1</b>
<b>DON</b>				
TDI <sup>4</sup> : 1000 $\text{ng}/\text{kg}$ bw/day				
Cereals for direct human consumption and cereal flours	750	221.9	GC 80	166425
Total ( $\text{ng}/\text{person}/\text{day}$ )				166425
Total ( $\text{ng}/\text{kg}$ bw/day) <sup>2</sup>				2377.5
% TDI				237.75 %
C ( $\text{ng}/\text{ml}$ )				166
<b>C<sub>used</sub> (<math>\text{ng}/\text{ml}</math>)</b>				<b>250</b>

<sup>1</sup> EFSA (2006)

<sup>2</sup> Assuming a 70 kg body weight adult

<sup>3</sup> Assuming that the tolerable weekly intake is equally spread over the week and that  $\text{TDI} = \text{TWI}/7 = 17.14$   $\text{ng}/\text{kg}$  bw/day

<sup>4</sup> SCF (2002)

**Table 2: Theoretical maximum daily intake (TMDI) and realistic intestinal concentration (C) of IMA and BEN estimated by a deterministic approach.** European maximal limits of residues (MRLs) in food specified in the Commission Regulation (EC) N°396/2005 of 23 February 2005 (amended by Commission Regulation (EC) N°839/2008 of 31 July 2008 for BEN and Commission Regulation (EU) N° 750/2010 of 7 July 2010 for IMA) and human European consumption data from GEMS/FOOD Regional Diets (WHO, 2003) ( $Q_{Ei}$ ) were used (ADI, acceptable daily intake; bw, body weight).

	Consumption data		IMA ADI <sup>1</sup> : 30 µg/kg bw/day		BEN ADI <sup>1</sup> : 100 µg/kg bw/day	
	$Q_{Ei}$ (g/person/day)	GEMS code	MRLs (mg/kg)	TMDI (µg/person/day)	MRLs <sup>2</sup> (mg/kg)	TMDI (µg/person/day)
<b>Cereals</b>						
Barley	19.8	GC 640	0.05	0.99	2	39.6
Maize	8.8	GC 645	0.05	0.44	0.01	0.088
Oats	2	GC 647	0.05	0.1	2	4
Rice	11.8	GC 649	0.05	0.59	0.01	0.118
Rye	1.5	GC 650	0.05	0.075	0.1	0.15
Wheat	178	GC 654	0.05	8.9	0.1	17.8
<b>Fruits</b>						
Citrus fruits	44.6	FC 1	5	223	0.5	22.3
Pome fruits	51.3	FP 9	2	102.6	0.2	10.26
Stone fruits	23.3	FS 12	0.05	1.165		
Apricots	3.5	FS 240			0.2	0.7
Cherries	3	FS 13			0.5	1.5
Peaches	12.5	FS 247			0.2	2.5
Plums	3.8	FS 14			0.5	1.9
Small fruits and berries	23.5	FB 18	0.05	1.175		
Table grapes	13.8	FB 269			0.3	4.14
Strawberries	5.3	FB 275			0.1	0.53
Blueberries	0.5	FB 20			0.1	0.05
Cranberries	0.3	FB 265			0.1	0.03
Currants	0.3	FB 21			0.1	0.03
Gooseberries	0.5	FB 268			0.1	0.05



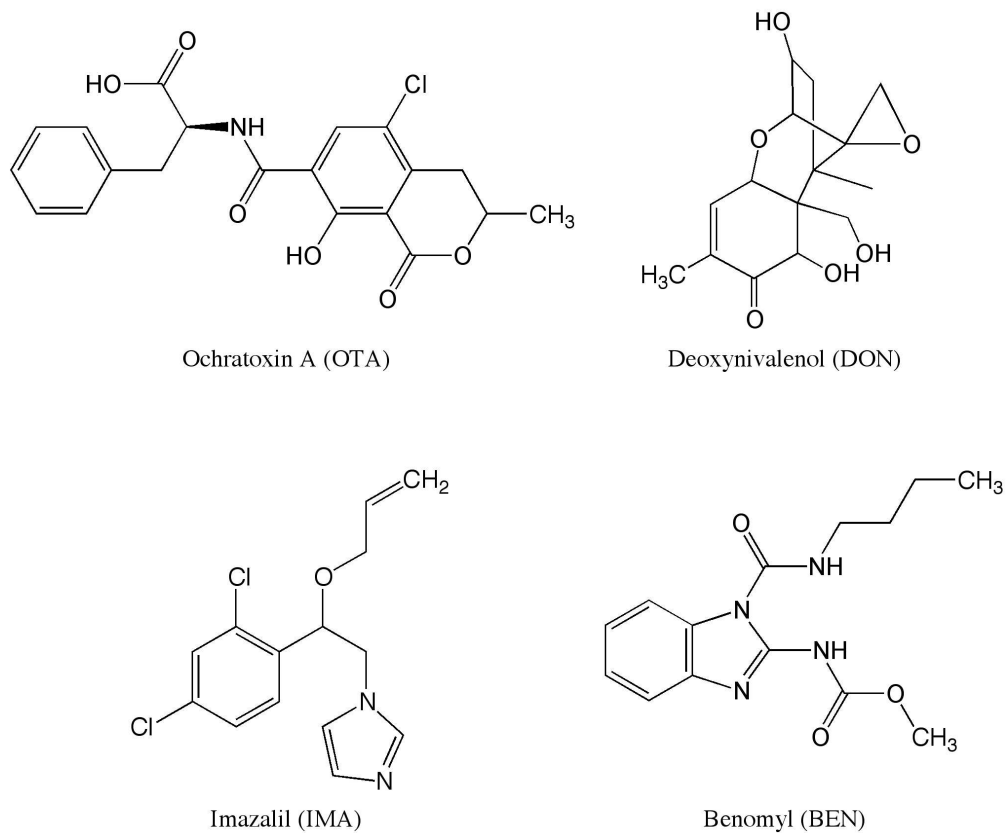
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5	Avocados	1	FI 326	0.05	0.05	0.1	0.1
6	Bananas	22.8	FI 327	2	45.6	0.1	2.28
7	Dates	0.3	FT 295	0.05	0.015	0.1	0.03
8	Figs	0.5	FT 297	0.05	0.025	0.1	0.05
9	Kiwi	1.5	FI 341	0.05	0.075	0.1	0.15
10	Olives	2.8	FT 305	0.05	0.14	0.1	0.28
11	Passion fruit	1.5	FI 351	0.05	0.075	0.1	0.15
12	Pineapples	3.3	FI 353	0.05	0.165	0.1	0.33
13	<b>Vegetables</b>						
14	Root and tuber vegetables						
15	Beetroot	2	VR 574	0.05	0.1	0.1	0.2
16	Carrots	22	VR 577	0.05	1.1	0.1	2.2
17	Parsnips	2	VR 588	0.05	0.1	0.1	0.2
18	Radishes	2	VR 494	0.05	0.1	0.1	0.2
19	Sweet potatoes	1.3	VR 508	0.05	0.065	0.1	0.13
20	Swedes	2	VR 497	0.05	0.1	0.1	0.2
21	Turnips	2	VR 506	0.05	0.1	0.1	0.2
22	Bulb vegetables	32.9	VA 35	0.05	1.645	0.1	3.29
23	Fruiting vegetables						
24	Tomatoes	66.6	VO 448	0.5	33.3	0.5	33.3
25	Peppers	10.4	VO 51	0.05	0.52	0.1	1.04
26	Aubergines	2.3	VO 440	0.05	0.115	0.5	1.15
27	Cucurbits						
28	Cucumber and gherkins	9	VC 424	0.2	1.8	0.1	0.9
29	Summer squash	3.5	VC 431	0.2	0.7	0.1	0.35
30	Melons	18.3	VC 46	2	36.6	0.1	1.83
31	Wintersquash	0.5	VC 433	0.05	0.025	0.1	0.05
32	Watermelon	7.8	VC 432	0.05	0.39	0.1	0.78
33	Sweetcorn	8.3	VO 447	0.05	0.415	0.1	0.83
34	<i>Brassica</i> vegetables						
35	Broccoli and cauliflower	13	VB 404	0.05	0.65	0.1	1.3
36	Brussels sprouts	2.7	VB 402	0.05	0.135	0.5	1.35
37	Chinese cabbage	0.1	VL 466	0.05	0.005	0.1	0.01
38	Kale	2	VL 480	0.05	0.1	0.1	0.2
39	Kohlrabi	0.1	VB 405	0.05	0.005	0.1	0.01
40	Leafy vegetables	51.7	VL 53	0.05	2.585	0.1	5.17
41	Legume vegetables	28.4	VP 60	0.05	1.42		
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Beans	12	VP 526			0.2	2.4
Peas	14	VP 63			0.2	2.8
<b>Stem vegetables</b>						
Asparagus	1.5	VS 621	0.05	0.075	0.1	0.15
Celery	2	VS 624	0.05	0.1	0.1	0.2
Leek	2	VA 384	0.05	0.1	0.1	0.2
Rhubarb	2	VS 627	0.05	0.1	0.1	0.2
Mushrooms	4	VO 450	0.05	0.2	1	4
<b>Pulses</b>	9.4	VD 70	0.05	0.47	0.1	0.94
<b>Nuts and oilseeds</b>	29.9	-	0.05	1.495	0.1	2.99
<b>Potatoes</b>	240.8	VR 589	3	722.4	0.1	24.08
<b>Stimulants</b>						
Tea	2.3	DT 171	0.1	0.23	0.1	0.23
Coffee beans	7.9	SB 716	0.1	0.79	0.1	0.79
Cocoa	3.1	SB 715	0.1	0.31	0.1	0.31
Chicory root	1	VR 469	0.05	0.05	0.1	0.1
<b>Sugar beet</b>	2	VR 596	0.05	0.1	0.1	0.2
<b>Hops</b>	0.1	DH 1100	0.1	0.01	0.1	0.01
<b>Animal products</b>						
Meat and offal	217.3	-	0.05	10.865	0.05	10.865
Eggs	37.6	PE 112	0.05	1.88	0.05	1.88
Milk	336.1	AO 31	0.05	16.805	0.05	16.805
Honey	1.3	-	0.05	0.065	1	1.3
Total ( $\mu\text{g}/\text{person}/\text{day}$ )				1223.3		234.456
Total ( $\mu\text{g}/\text{kg bw}/\text{day}$ ) <sup>3</sup>				17.47		3.34
% ADI				58.2%		3.34%
C (ng/ml)				1223		234
<b>C<sub>used</sub> (ng/ml)</b>				<b>2500</b>		<b>250</b>

<sup>1</sup> Set by JMPR (<http://www.inchem.org/pages/jmpr.html>)

<sup>2</sup> Sum of benomyl and carbendazim, expressed as carbendazim

<sup>3</sup> Assuming a 70 kg body weight adult



35 Figure 1: Chemical structure of food contaminants investigated in the present study: ochratoxin A  
36 (OTA), deoxynivalenol (DON), imazalil (IMA) and benomyl (BEN).  
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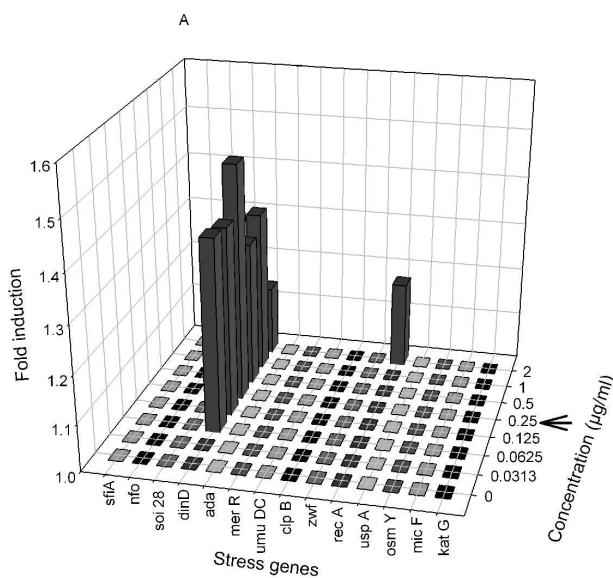
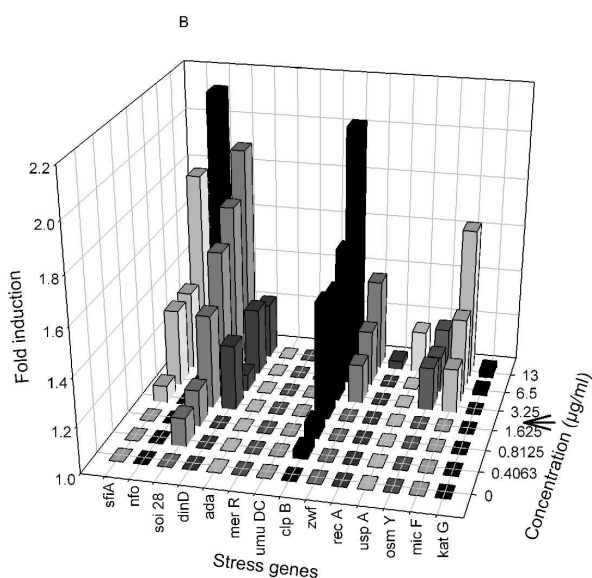


Figure 2: Prokaryotic gene expression profile after exposure of *E. Coli* strains to a concentration range of (A) DON (0.031 – 2 µg/ml) and (B) IMA (0.4 – 13 µg/ml). Values are means of three independent experiments performed in triplicates and are expressed as fold inductions, calculated with formulas as described in materials and methods. An arrow indicates the calculated realistic intestinal concentration. The different genes are responsive to: katG: oxidative stress, micF: membrane integrity and osmotic stress, osmY: osmotic stress, uspA: growth arrest, recA: SOS response, zwf: oxidative stress, clpB: protein perturbation, umuDC: radiation and/or chemically induced DNA damage, merR: heavy metals, ada: DNA damage (mainly methyl adducts), dinD: DNA damage, soi28: superoxide radical generating agents, nfo: single- and double-stranded DNA breaks and oxidative DNA damage, SfiA: SOS response to DNA damage.

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Figure 2: Prokaryotic gene expression profile after exposure of *E. Coli* strains to a concentration range of (A) DON (0.031 – 2 µg/ml) and (B) IMA (0.4 – 13 µg/ml). Values are means of three independent experiments performed in triplicates and are expressed as fold inductions, calculated with formulas as described in materials and methods. An arrow indicates the calculated realistic intestinal concentration. The different genes are responsive to: katG: oxidative stress, micF: membrane integrity and osmotic stress, osmY: osmotic stress, uspA: growth arrest, recA: SOS response, zwf: oxidative stress, clpB: protein perturbation, umuDC: radiation and/or chemically induced DNA damage, merR: heavy metals, ada: DNA damage (mainly methyl adducts), dinD: DNA damage, soi28: superoxide radical generating agents, nfo: single- and double-stranded DNA breaks and oxidative DNA damage, SfiA: SOS response to DNA damage.

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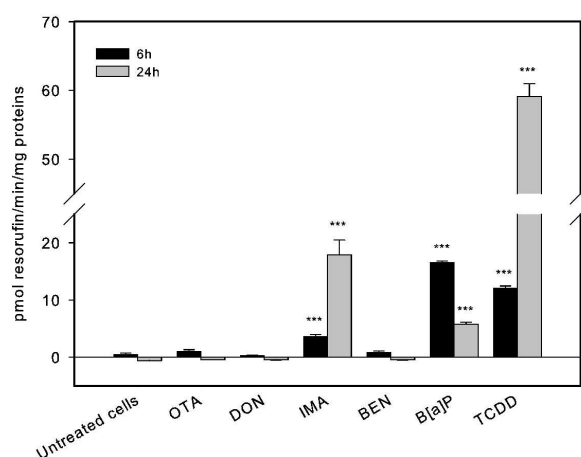


Figure 3: Modulation of CYP1A1 activity by OTA, DON, IMA and BEN in Caco-2 cells. Sixteen-day post-confluent cells seeded in 48-well plates (3.104 cells/cm<sup>2</sup>) were exposed to OTA (1 ng/ml), DON (250 ng/ml), IMA (2500 ng/ml) or BEN (250 ng/ml) for 6 or 24h. Positive controls consisted in cells treated with B[a]P (250 ng/ml) or TCDD (0.3 ng/ml) and negative control consisted in untreated cells. CYP1A1 activity was assessed through the measurement of the fluorescence produced by the conversion of 5  $\mu$ M 7-ethoxyresorufin to resorufin. Results are expressed in pmol resorufin produced/min/mg protein. Values are means  $\pm$  S.E.M. of three independent experiments performed in triplicates (n=9). \*\*\*P < 0.005 as compared to respective vehicle controls.

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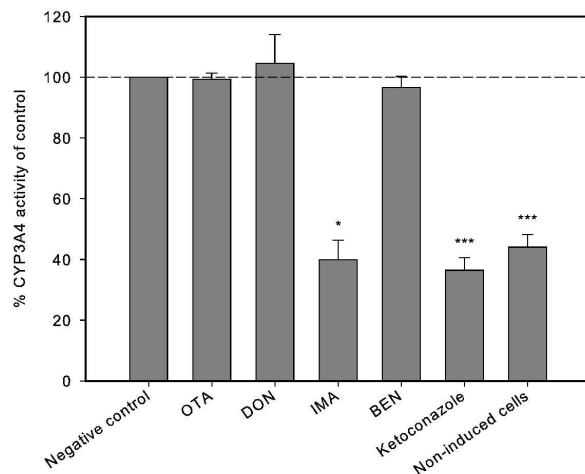


Figure 4: Modulation of CYP3A4 activity by OTA, DON, IMA and BEN in Caco-2 cells. Cells were seeded in 12-well plates (4.104 cells/cm<sup>2</sup>) and treated during 14 days from confluence with 0.5  $\mu$ M 1,25-vitD<sub>3</sub>, in order to induce CYP3A4 activity. Cells were then exposed for 3h simultaneously to testosterone (500  $\mu$ M) and to OTA (1 ng/ml), DON (250 ng/ml), IMA (2500 ng/ml), BEN (250 ng/ml) or ketoconazole (positive control) (26.6  $\mu$ g/ml). The CYP3A4 activity of the cells was then assessed through the HPLC determination of 6 $\beta$ -(OH)-testosterone in the cell medium. Non-induced cells represent the basal CYP3A4 activity without preliminary inductive treatment with 1,25-vitD<sub>3</sub>. Results are expressed in pmol 6 $\beta$ -(OH)-testosterone produced/min/mg protein and illustrated in % of negative control (5.13 $\pm$ 0.37 pmol/min/mg protein), consisting in untreated but pre-induced cells. Values are means  $\pm$  S.E.M. of three independent experiments performed in triplicates (n=9). \*P < 0.05, \*\*\*P < 0.005 as compared to respective vehicle controls.

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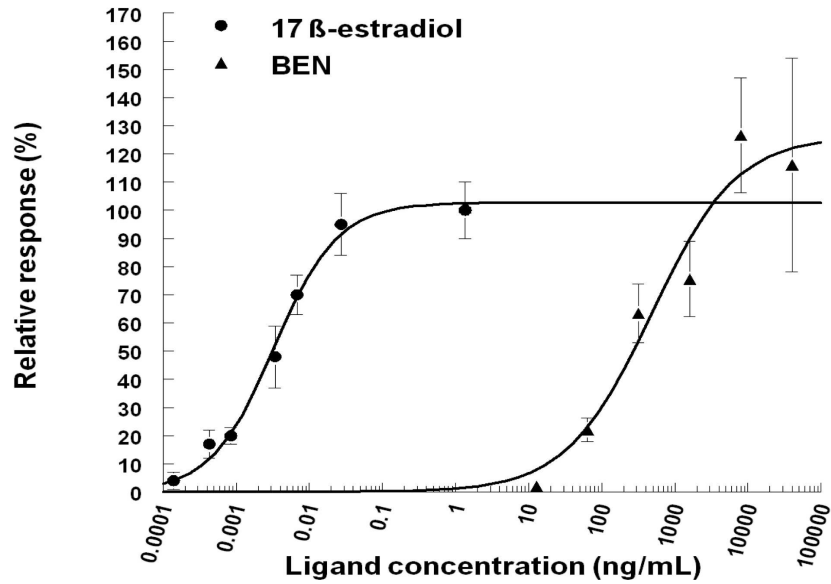


Figure 5: Dose-response curves of E2 and BEN in MMV-Luc cells. Exposure time was 24h. Results are expressed as means of the relative response (%)  $\pm$  S.E.M. (100% with highest tested concentration of E2), of two independent experiments performed in triplicates (n=6), in function of the ligand concentration (ng/ml).  
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