ARYL HYDROCARBON RECEPTOR-MEDIATED AGONIST/ANTAGONIST/SYNERGIC ACTIVITIES OF FOOD POLYPHENOLS ARE SPECIES- AND TISSUE-DEPENDENT

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Abstract
Aryl hydrocarbon Receptor (AhR) is a ligand-activated transcription factor mediating the adverse effects of dioxins and Polycyclic Aromatic Hydrocarbons (PAHs). In this study, we investigated the species- and tissue-dependent AhR agonistic/antagonistic activities of 3 food polyphenols (quercetin, chrysin and genistein). HepG2, T47D and H4IIE cell lines were used in luciferase reporter gene assays. Human hepatoma (HepG2) and human breast tumour (T47D) cells were compared for the tissue-dependent effect. Rat hepatoma (H4IIE) and human hepatoma (HepG2) cells were compared for the species-dependent activity. We concluded that quercetin, chrysin and genistein act in a species- and tissue-specific manner. Indeed, they suppress the response induced by 2, 3, 7, 8 tetrachlorodibenzo-*p*-dioxin (TCDD) in HepG2, but not in H4IIE and T47D cells. In contrast, they act as AhR agonists in T47D cells. Furthermore, genistein and chrysin act in synergy with 3-methylcholanthrene (3-MC) in T47D cells. The flavonoids/TCDD concentration ratios used in the HepG2 and T47D cell-based assay were close to those found in the serum of humans, taking into account their diet and the background contamination by dioxins. These results suggest that a vegetable-based diet could reduce or increase the possible dioxin toxicity associated to food intake, depending of the target tissue.

Introduction
Dioxins, such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAHs) induce a broad spectrum of adverse effects known to be mediated by the Aryl hydrocarbon Receptor (AhR), a transcription factor ligand-dependent. Cytochrome P450 1A1 (CYP1A1) monooxygenase is one of the xenobiotic-metabolizing enzymes induced by these compounds through activation of AhR[1]. Besides its physiological role in the detoxification of xenobiotic compounds, the activity of this enzyme can be deleterious since it generates mutagenic metabolites and reactive oxygen. Aryl hydrocarbons such as TCDD and 3-methylcholanthrene (3-MC) are known to be inducing ligands of AhR. Flavonoids belong to the group of polyphenolic compounds that are widely distributed in all foods of plant origin[2] and are also able to bind AhR[3]. Interest in the possible health benefits of flavonoids has increased owing to their antioxidative activity, anticarcinogenicity and the inhibition of several enzymes including protein kinases and cytochrome P450s. Some flavonoids can act as antagonists (inhibition) or agonists (activation) of the AhR[4,5]. We investigated the agonistic/antagonistic effects mediated by AhR of three flavonoids largely studied; a flavone (chrysin), a flavonol (quercetin) and an isoflavone (genistein), using cell-based luciferase reporter gene assays. In order to determine the species- and tissue-dependent effect of the flavonoids, four stably transformed cell lines were used in luciferase reporter gene assays: three were transformed with a reporter vector containing four dioxin-responsive elements (DREs) upstream of the thymidine kinase promoter and the luciferase gene (HepG2, T47D and H4IIE). H4IIE DR-CALUX® cells (containing part of the mouse CYPA1A promoter) were also studied to compare two H4IIE cell lines transformed with luciferase reporter vectors containing different promoters. Rat liver cancer H4IIE and human liver cancer HepG2 cells were compared for the species dependent activity. Human liver cancer HepG2 were compared to human breast cancer T47D cells for the tissue dependent effect.

Materials and Methods
Stable reporter cell lines. For our experiments, we used four stably transformed dioxin-responsive cell lines. Rat hepatoma H4IIE DR-CALUX® cells were purchased from BioDetection System (Amsterdam, Nederlands). These cells were stably transfected with a construct containing the upstream region of the mouse CYP1A1 gene (from -1301 to -819) harbouring four Dioxin Responsive Elements (DREs), the MMTV viral promoter and the luciferase gene as described by Garrison and co-workers[6]. T47D (human breast tumour, ATCC number: HTB-133), HepG2 (human hepatoma, ATCC HB-8065) and H4IIE (rat hepatoma, ATCC CRL-1548) were obtained

from the American Type Culture Collection (ATCC, Manassas, VA) and stably transfected with 4 DREs (5' GGGTCCCAGTGCTGTCACGCTAG 3'- a DRE consensus sequence), in vitro synthesised, upstream of the thymidine kinase promoter and the luciferase reporter gene.. Cells were grown in 75 cm^2 culture flasks in D-MEM medium (Dulbecco's Essential Medium), MEM medium (Minimum Essential Medium) and MEM alpha medium (Minimum Essential Medium), respectively, supplemented with 10% heat-inactivated fetal bovine serum, at 37°C in 5% $C0_2$.

Cell-based assay for Aryl hydrocarbon Receptor (AhR)–mediated agonistic/antagonistic activities For the identification of agonistic/antagonistic AhR activities, a cell-based luciferase reporter gene assay was used[6]. The cell-based assay for agonistic AhR activity was carried out as follows. Dioxin-responsive cells were cultured in 96-well culture plates, and flavonoids were added at final concentrations of 2.5, 5, 10, 20 and 40 µM (quercetin and chrysin were dissolved in DMSO and genistein was dissolved in ethanol). The solvent concentration was 0.4 % in the culture medium. A dose-response curve with TCDD was performed on the same plate. The plates were incubated at 37°C in 5 % CO_2 during 24 h. After incubation, the cell viability was confirmed under a microscope and with a MTT (MiTochondrial Test) assay[7]. Subsequently, the medium was removed and the cells were lysed. After the addition of luciferin, the luciferase activity was determined using a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany), and reported as Relative Light Units (RLUs). RLUs data have then been transformed in relative response (percent of the maximal RLU response to TCDD). The maximal relative response (fixed to 100%) was obtained with 300 pM TCDD for both H4IIE (DR-CALUX® and ULg) cell lines and 10 nM TCDD for HepG2 and T47D cell lines. The cell-based assay for antagonistic AhR activity was carried out with a constant concentration of inducer (TCDD or 3-MC) and increasing concentrations of flavonoids (2.5, 5, 10, 20 and 40 µM). TCDD concentrations were 300 pM and 10 nM for rat and human cells respectively. 3-MC concentration was 2.5 µM and 20 µM for HepG2 and T47 rat and human cell respectively. The solvent concentration was 0.8% in the culture medium.

Results and Discussion

Aryl hydrocarbon Receptor (AhR) – mediated agonistic activities. The flavonoids tested had no AhR-mediated agonistic activities in hepatoma cells, neither in rat (both DR-CALUX® and ULg H4IIE), nor in human cells (HepG2) (data not shown). In contrast, in human breast tumour (T47D), chrysin and quercetin induced a high response. The concentrations producing 25% of the maximal response induced by TCDD (EC_{TCDD25}) were 30 pM for TCDD and 3.6 and 8.7 µM for chrysin and quercetin, respectively. As shown in figure 1, 20 µM chrysin induced a higher response than 10 nM of TCDD (relative response of 145%) and the maximal luciferase activity induced by quercetin was 61% of the maximal response to TCDD. The AhR-mediated response to genistein, in these cells, was very weak and increased with concentration up to 18 % of relative response.

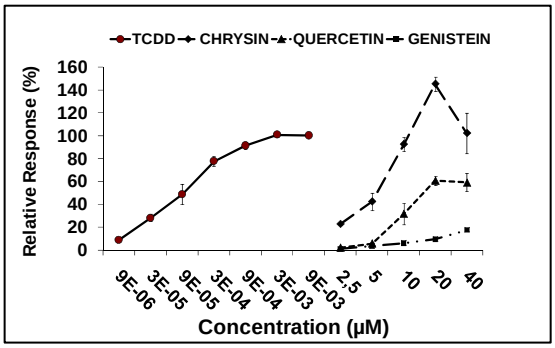

Figure 1: Ah Receptor-mediated agonistic activities measured in stably transfected T47D human breast tumour cells. TCDD and flavonoid dose-response curves were compared. Relative response: percentage of the response (luminescence) relative to that obtained with 10 nM TCDD. Results are expressed as mean ± SD (n=3 independent experiments).

Aryl hydrocarbon Receptor (AhR) – mediated antagonistic activities in presence of TCDD.

Experiments were carried out using concentrations of TCDD producing the highest luciferase activity (see Materials and Methods). As shown in Figure 2A, quercetin had no significant AhR-mediated antagonistic activity in rat hepatoma (H4IIE DR-CALUX® or ULg) and in human breast tumour cells (T47D). For these cell lines, only a weak inhibition of luciferase activity was observed at concentrations of 20 and 40 µM of quercetin. In HepG2 human hepatoma, a significant inhibition of TCDD-induced response was obtained with 20 µM and 40 µM of quercetin, which almost abolished the response to TCDD (only 10% of the TCDD response remained). The same kind of results were obtained with chrysin (figure 2B), with no AhR-mediated antagonistic activity observed at the concentrations tested neither in H4IIE rat hepatoma (both DR-CALUX® and ULg), nor in human

breast tumour cells (T47D), while the TCDD-induced response was inhibited in HepG2 human hepatoma at chrysin concentrations of 20 µM and 40 µM. When the cells are exposed to both TCDD and genistein, the pattern of activity is different (figure 2C). Like quercetin and chrysin, genistein displayed AhR-mediated antagonistic activity only in human hepatoma cells (HepG2), with a decrease of the TCDD activity down to 46% of the TCDD maximal response. In contrast, in human breast tumour cells (T47D), genistein and TCDD seem to display a synergistic activity on the AhR-mediated transactivation, since we observed an increase of the relative response up to 208% at 40 µM genistein. MTT assays were performed on HepG2, H4IIE (both DR-CALUX® and ULg) and T47D with quercetin, chrysin and genistein to assess the flavonoid's toxicities on the cells. Results reveal that flavonoids do not affect the viability of these cell lines (data not shown), showing that the flavonoid's AhR-mediated antagonistic activities in HepG2 cells are not due to cytoxicity.

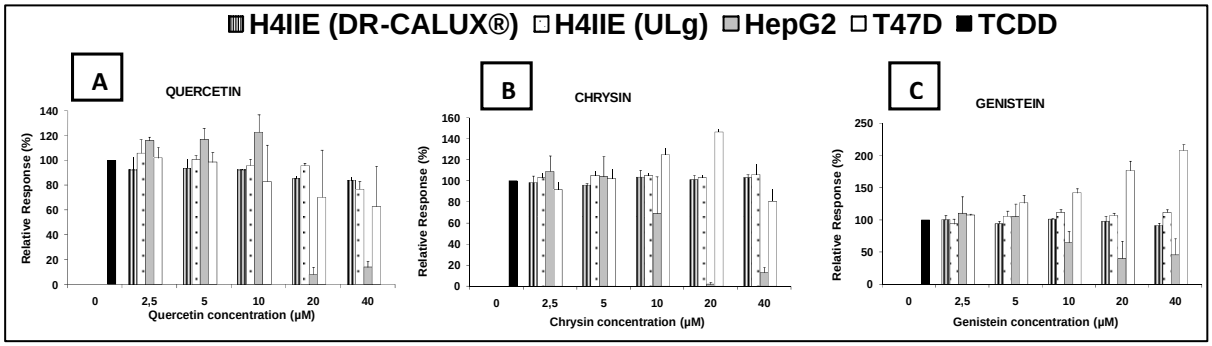

Figure 2: Ah Receptor-mediated antagonistic activities of quercetin (A), chrysin (B) and genistein (C) in presence of TCDD as AhR inducer in four stably transfected cell lines. T47D and HepG2 cells were treated with 10 nM of TCDD in combination with increasing concentrations of quercetin (A), chrysin (B) and genistein (C). H4IIE (DR-CALUX® or ULg) cells were treated with 300 pM of TCDD in combination with increasing concentrations of quercetin (A), chrysin (B) and genistein (C). Relative response: percentage of the response obtained with TCDD. Results are expressed as mean ± SD (n=3 independent experiments).

Aryl hydrocarbon Receptor (AhR) – mediated antagonistic activities in presence of 3-MC.
Experiments were carried out using concentrations of 3-MC producing about the same induction than TCDD (see Materials and Methods). As shown in Figure 3, the flavonoids tested act in synergy with the 3-MC in human breast tumour (T47D). Indeed, production of luciferase was increased with the three flavonoids in different manners. Genistein induced a six-fold higher production of luciferase than that induced by 3-MC alone. The synergy seems to be weaker with chrysin since luciferase production was only 4-fold higher at 10 µM of chrysin. For quercetin, synergy was very weak with a maximal induction of 154% of the maximal response to 3-MC at 5 µM. In HepG2 human hepatoma cells (Figure 4), no AhR-mediated antagonistic activity was observed with quercetin and chrysin, while genistein seems to act in synergy with the 3-MC. Indeed, an increased production of luciferase was observed (179% of the 3-MC induction) at 40 µM genistein. In H4IIE cells, no AhR-mediated antagonistic or synergistic activity was observed with flavonoids (data not shown). We conclude that genistein and 3-MC have synergistic activities on the AhR-mediated transactivation stronger in T47D than in HepG2 cells and that chrysin and 3-MC have synergistic activities on the AhR-mediated transactivation only in T47D cells.

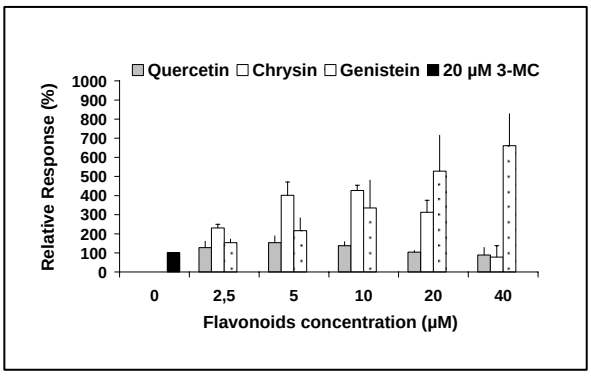

Figure 3: Ah Receptor-mediated antagonistic activities of quercetin, chrysin and genistein in presence of 3-MC as AhR inducer in human breast tumour (T47D).

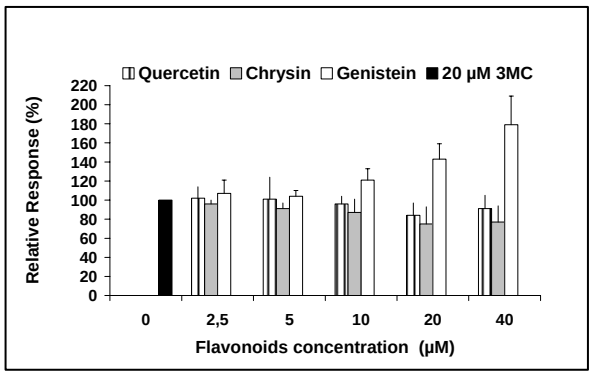

Figure 4: Ah Receptor-mediated antagonistic activities of quercetin, chrysin and genistein in presence of 3-MC as AhR inducer in human hepatoma cells (HepG2).

In conclusion, we have demonstrated that the flavonoids tested here act in a species- and tissue-dependent manner. Indeed, quercetin, chrysin and genistein suppress the TCDD-induced response at concentrations of 20 µM and 40 µM in HepG2 human hepatoma cells, act as agonist in T47D human breast tumour and have no effect in H4IIE rat hepatoma (both ULg and DR-CALUX®). Surprisingly, flavonoids do not suppress the 3-MC-induced response in HepG2, suggesting another mechanism compared to TCDD in these cells.
Curiously, in T47D cells, genistein and chrysin act in synergy with 3-MC and TCDD. These results have not been reported in the literature. An explanation could be that T47D cells express the Estrogen Receptor to which the genistein could bind[8]. Furthermore, chrysin (data not published) and 3-MC[9] induce estradiol-responsive genes. An AhR-ER cross-talk, as previously reported[9,10], could contribute to the synergy observed in these cells.
CYP1A1, a metabolizing enzyme, is expressed after activation of AhR by its ligand and may produce carcinogenic compounds. The concentration ratios of flavonoids/TCDD (10^{-4}) used in the cell-based reporter gene assay using human hepatoma HepG2 and human breast tumour T47D cells were close to those found in the serum of humans[5] (10^{-4} to 10^{-6}), taking into account their diet and the background contamination by dioxins. These results suggest that a vegetable-based diet could, depending on the tissue considered, either reduce or increase the possible dioxin toxicity mediated partly by CYP1A1 associated to the food intake. This study demonstrates that ingestion of different compounds in the diet, such as flavonoids and TCDD, may result in various effects, antagonistic or synergistic. Toxicological assessment and risk analysis of chemicals are generally performed on unique substances. Nevertheless, it is well known that a simple additive model is generally not applicable to mixtures of active substances. Synergistic or antagonistic effects are often observed. These kinds of studies must be considerably developed in the future to increase our knowledge on mixture toxicology.

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