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Bioassay directed identification of natural aryl hydrocarbon-receptor agonists in marmalade

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ABSTRACT

Citrus fruit and citrus fruit products, like grapefruit, lemon and marmalade were shown to contain aryl hydrocarbon receptor (AhR) agonists, as detected with the DR CALUX® bioassay. This is of interest regarding the role of the Ah-receptor pathway in the adverse effects of dioxins, PCBs and other aromatic hydrocarbons. So far it is unclear which compounds in citrus fruit are responsible for the AhR-mediated activity and whether regular exposure to these compounds can cause effects comparable to, e.g. dioxins.

The present study aimed at developing a method for identifying unknown Ah-receptor agonists in citrus products based on bioassay directed analysis, using marmalade as a first target. Following extraction with hexane and purification on an aluminium oxide-column, the extract was fractionated by HPLC using a C-18 semi-preparative column. Fractions were extracted, solvent-exchanged into dimethylsulfoxide and subsequently tested with DR CALUX®. Extracts were shown to contain primarily coumarins, furocoumarins (FCs) and polymethoxyflavones (PMFs). Identification of fractions most active in the bioassay via LC/MS revealed that bergapten (an FC) is the most important Ah-receptor agonist in marmalade. The approach and method developed resulted in the successful identification of the bioactive component. However, potential pitfalls of the procedure will be discussed.

1. Introduction

Since ancient times, bioassays have been the most reliable way to detect known and thus far unknown toxins. Food tasters were employed to decrease the risks of poisoning by enemies and also today, rodents are used to ensure, e.g. the absence of marine toxins in shellfish. New emerging risks were often detected based on their effects in animals, like aflatoxins causing turkey X-disease [1] and dioxins causing chicken edema disease [2]. Also incidents are normally discovered by their adverse effects in animals, as shown during the accidental mixing of a flame retardant mixture into cattle feed in Michigan in 1975 [3], the Belgian dioxin incident in 1999 [4], and the presence of medroxyprogesterone (MPA) in pig feed in the Netherlands in 2002 [5]. Of course, the deliberate use of animals for testing of food is highly undesirable. Bioassays based on mammalian cell-lines, yeast or bacteria offer an interesting alternative and allow the rapid screening of large numbers of food or feed samples for the presence of known and unknown agonists with certain adverse effects. The use of the E-screen, an in vitro test for estrogenic properties, resulted, e.g. in the discovery of the estrogenic potency of nonyl-phenol [6]. A yeast-based estrogen assay detected the presence of 17β-estradiol in the pig feed contaminated with MPA [7].

The DR CALUX® bioassay is an assay developed for the screening of samples for dioxins and dioxin-like compounds

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[8,9]. Following binding of the target compounds to the so-called Ah-receptor, the transport of the receptor–ligand complex into the nucleus and the binding to a dioxin responsive element in the DNA, the cells respond with an increased and dose-related production of luciferase. Binding to the Ah-receptor and the resulting up- and down-regulation of a number of genes is thought to underlay the toxic effects of dioxins and dioxin-like PCBs. In principle the test can also respond to many other Ah-receptor agonists but the specificity for dioxins and dioxin-like PCBs is increased by the clean-up procedure using acid silica [10]. Nevertheless, the testing of samples results in a certain number of false-positive results. Furthermore, extracts of many food items, not purified on acid silica, have actually been shown to cause a positive effect in this assay [11,12]. Although the slow degradation and accumulation of dioxins probably discriminates them from many natural AhR-agonists present in food, the question arises whether a frequent exposure to such compounds may cause AhR-mediated adverse effects. Increased levels of unknown AhR-agonists in blood have been reported [13,14] and might be explained by such natural compounds. It is therefore important to identify the responsible components in order to further examine their effects in animals and humans.

The present study aimed at developing a bioassay directed approach for the identification of natural AhR-agonists (NAhRAs) in citrus products based on the fractionation of extracts and retesting with the bioassay. Compounds identified in extracts and purified fractions were purchased and tested in the bioassay to confirm their contribution to the response in the bioassay.

2. Materials and methods

2.1. Chemicals

The pGudLuc 1.1-transfected H4IIE cells were obtained from the Department of Toxicology, Agricultural University, Wageningen. Eagle's Minimum Essential Medium Alpha (α-MEM), penicillin/streptomycin (P/S), 2-methyl-2-butene (amylene), β-hydroxytoluene (BHT) and bergapten (5-methoxypsoralene), 99% pure, from Sigma–Aldrich (Zwijndrecht, The Netherlands), foetal bovine serum (FBS) from Invitrogen (Breda, The Netherlands), *n*-hexane, diethyl ether, acetonitrile, dichloromethane and methanol from Biosolve B.V. (Valkenswaard, The Netherlands), anhydrous sodium sulphate, aluminum oxide and dimethylsulfoxide (DMSO) from Merck (Darmstadt, Germany), bergamottin, heptamethoxyflavone, imperatorin, isoimperatorin, isopimpinellin, nobiletin, psoralen, sinensitin, tangeretin and tetra-*O*-methylscutellarein from APIN chemicals Ltd. (Oxon, United Kingdom), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Marmalade, grapefruit and lemon were purchased from a local supermarket.

2.2. Extraction and purification of the samples

Ten millilitres of juice or 5 g of marmalade were suspended into 20 mL methanol/water 85/15 (v/v) and extracted twice with 20 mL hexane/diethyl ether 97/3 (v/v) [15]. The hexane/diethyl ether extracts were divided into aliquots containing the equivalent of 2 mL of juice or 1 g of marmalade each. The extracts were evaporated till approximately 50 μL and subsequently mixed with 1 mL hexane, in order to exclude diethyl ether.

Empty filtration SPE columns (Bakerbond SPE) containing a filter at the bottom, were packed with 1 g aluminium oxide containing 14% water, and some anhydrous sodium sulphate on top. The aluminium oxide columns were conditioned subsequently with 4 mL dichloromethane, 4 mL dichloromethane/hexane 50/50 (v/v) and 4 mL hexane. The extracts were applied to the columns and subsequently eluted with 4 mL hexane, to elute mostly lipophilic compounds, and with 4 mL dichloromethane, to elute the compounds of interest, as shown by the activity in the DR CALUX®-assay. The hexane and dichloromethane subsamples were pooled for each sample and solvent exchanged into 40 μL of DMSO by nitrogen evaporation.

Twenty microlitres was mixed with 2 mL culture medium and used to expose the pGudLuc 1.1-transfected H4IIE cells (DR CALUX®).

2.3. HPLC-fractionation of the sample extracts

HPLC fractionation of extracts was performed on a Waters HPLC system (Waters 717 plus autoinjector, 510 and 515 pumps and a Waters 996, Photodiode Array Detector). To prevent false positive outcome of fractions in the DR CALUX® due to either unknown active compounds eluted from the LC-18 db column or substances introduced during the extraction procedure, a blank sample was injected and fractionated before each citrus extract. This blank sample was subjected to the same clean-up procedure as described for the sample extracts. The HPLC procedure was as follows: a 100 μL aliquot of blank or extract solution (20 μL DMSO extract mixed with 1 mL acetonitrile/water 50/50 (v/v)) was injected and separated on a Supelco LC-18 db semi-preparative column (250 mm × 4.6 mm I.D.). Solvent A was water/acetonitrile (95:5) and solvent B acetonitrile. The following gradient was applied: 0–20 min, 40–80% of B (linear), 20–25 min, 80–100% B (linear), 25–35 min, 100% B, 35–40 min from 100% to 40% B (linear), 40–55 min, 40% of solvent B. The total run time was 55 min and the flow rate was 0.8 mL min^{-1}. The system was coupled to a photodiode array detector (PDA) set at 200–600 nm. Fractions collected were extracted twice with dichloromethane. The dichloromethane was reduced to a small volume under vacuum, than mixed with 20 μL DMSO and further evaporated under nitrogen. To the remaining DMSO extract, 500 μL plain culture medium (α-MEM without FBS and P/S (α-MEM (−)) was added. Of this solution, 100 μL was re-injected on the HPLC system to check the recovery of compounds during the fractionation. To the remaining 400 μL, 400 μL culture medium (α-MEM with 20% FBS, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) was added and used to expose the pGudLuc 1.1-transfected H4IIE cells (DR CALUX®).

2.4. Testing of standards

Purchased standards were dissolved in DMSO and further diluted in DMSO to obtain the required concentrations. An