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Application of the equivalency factor concept to the phototoxicity and –genotoxicity of furocoumarin mixtures

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ABSTRACT

Furocoumarins (FCs) are natural constituents widely occurring in plants used as food or in phytomedicines, cosmetics, etc. Some FCs exert dermal photo-toxicity and -genotoxicity when combined with UVA irradiation. For a few congeners, skin tumor formation has been described in humans and laboratory animals. Since almost no information is available on the photo-toxic properties of several congeners, we analyzed the photo-cytotoxic, photo-mutagenic, and photo-clastogenic properties in V79 cells for thirteen naturally occurring FCs, and for the coumarin limettin. Furthermore, nine FC mixtures including one mixture based on the FC pattern of an *Angelica archangelica* extract were tested in the same assays. We found that the concept of relative potency factors for photo-cytotoxic, -mutagenic, and -clastogenic-potencies of FCs, setting the value for 5-methoxypsoralen at 1.00, was applicable to all congeners tested. The concept was used successfully to describe the photo-toxic properties of binary mixtures of 5- and 8-methoxypsoralen. Furthermore, the photo-genotoxic (photo-mutagenic and -clastogenic) properties of complex FC mixtures comprising up to nine different congeners could be predicted. These data suggest that FCs can differ widely in their photo-toxic and photo-genotoxic properties but show relatively strict additivity with respect to their on target-effects when occurring as complex mixtures.

1. Introduction

Furocoumarins (FCs) are secondary metabolites naturally occurring in a large variety of plants including a number of species used as food, or for ingredients in cosmetics, flavourings or herbal medicines (Santana et al., 2004; SKLM, 2006). Plants are considered to synthesize these compounds as part of their chemical defence strategy in order to protect e.g. roots or fruits against microbial infections (Ostertag et al., 2002). Upon UVA irradiation, FCs can undergo photo-activation which makes them highly reactive towards cellular target molecules such as proteins or nucleic acids (Gasparro et al., 1997). This mode of action is the reason for the outstanding photo-toxicity and the proven photo-carcinogenicity of certain FC congeners, e.g., in human skin (IARC, 1986). Furthermore, certain dietary FCs have been shown to act as highly potent inhibitors of a number of cytochrome P450 (CYP) monooxygenases (Guo and Yamazoe, 2004). This property, being independent of photo-activation, has led to wide-spread recommendations to avoid FC-rich food items, in particularly, grapefruit juice when taking certain types of medication (Baumgart et al., 2005; Edwards et al., 1996; Fukuda et al., 1997).

However, knowledge on photo-toxic and photo-genotoxic properties is limited to less than a dozen congeners, while over ninety congeners have been described to occur in plants (Scott et al., 1976). Furthermore, usually several different FCs occur in plant materials rising the issue of combination effects, interactions, etc. Although the mechanisms of action of FCs have been elucidated decades ago, the recent need for evaluation of FC mixtures and of more rarely occurring congeners has not been addressed.

Recently, we have published an approach to describe the photo-(cyto)toxicity, -mutagenicity, and -clastogenicity of FC congeners using a test battery in V79 cells irradiated with a defined UVA dose (Lohr et al., 2010; Raquet and Schrenk, 2009). The potency factors termed relative photo-(cyto)toxicity, photo-mutagenicity and photo-clastogenicity factors (RPTF, RPMF, RPCF) describe the potencies of a certain congener in relation to 5-methoxypsoralen (5-MOP) the most potent congener in V79 cells in all three test systems.

Here, we apply the aforementioned test battery to a broader variety of FCs and investigate the effects of a number of various mixtures of FCs in order to analyse possible interactive effects, which may be relevant within a more realistic exposure scenario.

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2. Materials and methods

2.1. Materials

The V79 Chinese hamster cell line was a generous gift from Dr. Gerhard Eisenbrand, Food Chemistry and Toxicology, University of Kaiserslautern, Germany.

Angelicin, 6′,7′-dihydroxybergamottin, limettin and 8-methoxypsoralen (8-MOP) were purchased from Sigma–Aldrich (Schnelldorf, Germany), bergamottin, imperatorin, isopimpinellin, khellin and psoralen from Roth (Karlsruhe, Germany), bergaptol, byakangelicin, isobergapten, oxypeucedanin hydrate, phellopterin, pimpinellin, sphondin and xanthotoxol from Herboreal Ltd. (Edinburgh, United Kingdom), epoxybergamottin, heraclenin, isoimperatorin and oxypeucedanin from Phytolab (Vestenbergsgreuth, Germany), and 5-methoxypsoralen (5-MOP) from TCI Europe (Zwijndrecht, Belgium). An alcoholic extract of roots of *Angelica archangelica* was a generous gift from Dr. O. Kelber, Steigerwald Arzneimittelwerke, Darmstadt, Germany. Pencillin/streptomycin solution (100×) and fetal calf serum were from PAA, Cölbe, Germany. All other chemicals were of the highest purity commercially available.

2.2. Cell culture, treatment and cytotoxicity testing

V79 cells were cultured under sterile conditions in a humidified atmosphere under air (5% CO_2) at 37 °C. Irradiation was carried out at a UVA dose of 125 mJ/cm^2, furocoumarins were dissolved in DMSO and were added to the culture groups as described previously (Raquet and Schrenk, 2009), the alcoholic extract of *A. archangelica* roots was used as received for cell culture treatment. Controls received DMSO only, either with or without UVA treatment. With or without a short UVA treatment, cytotoxicity of furocoumarins was tested 20 h (bergamottin) or 3 days later using the Alamar Blue™ assay as described (Lohr et al., 2010).

2.3. HPRT assay

HPRT gene mutations were analyzed in V79 cells as previously described (Lohr et al., 2010). Briefly, 7.7×10^5 cells were seeded in 10 ml N-medium (500 ml DMEM low glucose, 56 ml fetal calf serum, 5.6 ml penicillin/streptomycin solution) on 94 mm Petri dishes and incubated under standard conditions for 24 h. Then, medium was replaced by 10 ml fresh S-medium (500 ml DMEM low glucose, 5 ml penicillin/streptomycin solution) with the test compounds added, and cultures were incubated for 30 min. MNNG (20 μM) was used as positive control. The test compounds were added dissolved in DMSO (50 μl/plate), controls were treated with DMSO only. After the incubation period, medium was removed, cultures were rinsed with PBS, and irradiated with UVA at various doses. Then, 10 ml N-medium was added, and the cultures were incubated. After 24 h, the cells were trypsinized, counted, and 10^6 cells were seeded in 15 ml N-medium in culture flasks. After additional 48 h of incubation, this procedure was repeated twice. Then, cells were trypsinized, centrifuged, and re-suspended in 8 ml N-medium and counted. Afterwards, 240 cells were seeded in Petri dishes (duplicates) in order to determine the cloning efficiency (CE). 10^6 Cells were seeded in 15 ml TG-medium (6-thioguanin containing selection medium) in culture flasks (triplicates). After nine more days in culture, clones as well as CE-colonies were stained with methylene blue and counted. Mutation frequency was calculated as described previously (Raquet and Schrenk, 2009).

2.4. Micronucleus assay

V79 cells were seeded in 4 ml N-medium on 60 mm cell culture dishes (10^5 cells per dish), and were kept in culture for 24 h under standard conditions (see above). Then, medium was replaced by fresh S-medium (4 ml), containing furocoumarins (or limettin) dissolved in 20 μl DMSO, and cultures were incubated over 30 min. Then, medium was removed, cultures were rinsed with PBS and irradiated with UVA at various doses. Negative controls received DMSO only, positive controls were treated (without irradiation) with mitomycin C (0.1 μg/ml) in 4 ml N-medium for 24 h. Furthermore, the highest concentrations of FCs and limettin were also tested without subsequent irradiation. Then, the medium was removed, the cultures were rinsed with PBS, and incubated with 4 ml medium for another 20 h.

Then, medium was removed, the cultures were rinsed with PBS, fixed with 3 ml cooled Carnoy's solution and kept at −20 °C for at least 20 min. Superficial Carnoy's solution was then removed, and 3 ml DAPI staining solution were added and incubated at −20 °C for 10 min. Then, the staining solution was removed, the dishes were washed with 2 ml methanol, and air-dried. The fixed and stained cultures were inspected microscopically by counting the micronuclei in 10^3 cells/plate in a blinded (coded) manner.

2.5. Calculation of effect parameters

The P(X)EF, i.e. PTEF, PMEF, and PCEF values were calculated from the slopes of the linear concentration–response curves for the various furocoumarins at a UV-dose of 125 mJ/cm^2 according to

$$P(X)EF_{Furocoumarin} = \frac{Slope_{Furocoumarin}}{Slope_{5-MOP}}$$

as described in detail in Raquet and Schrenk (2009). For binary mixtures of 5-MOP and 8-MOP, their behavior in mixtures was analyzed graphically: In each graph, the results of various mixtures of 8-MOP and 5-MOP plus UVA resulting in the same effect were regarded, e.g. the induction of a mutation frequency of 50×10^{-6}. The concentration of 8-MOP was plotted as a function of the 5-MOP concentration, the so-called isobologram, displaying an iso-effect line. In case of linearity of this isobole, an additive behavior of the components can be assumed, whereas a convex line would indicate antagonistic effects, a concave one synergistic behavior. For mixtures of $n > 2$ components, this method can be generalized to the 'interaction index' (CI): $\sum_{i=1}^{n} d_i/D_i = 1$ with d_i = combined equivalents of d at $i = 1$ to n components resulting within the mixture in a certain total effect, and D_i = concentrations of the reference compound(s) at $i = 1$ to n, leading to the same effect level (Berenbaum, 1981). A CI value of 1 indicates additivity, a CI-value > 1 antagonism, and a CI < 1 synergism (Groten et al., 2001; Carter, 1995). To analyze the effects of more complex mixtures, and hence the possible interaction of the components, we used certain concentration levels of the mixture, calculating the equivalents via the PCEF/PMEF/PCEF model (using the values calculated on a mass basis), and comparing it to the actually measured effect level. If this calculation is carried out for various levels of the same mixture (identical relative levels of the components), a slope and confidence intervals can be calculated based on linear regression analysis.

2.6. Statistical analysis

All data are given as means ± standard deviation of >3 independent experiments if not stated otherwise, i.e. ≥3 different passages of V79 cells were used. For the calculation of EC_{50} values, linear regressions and 95% confidence intervals, OriginPro 7.5 (Microcal, Northhampton, USA) was used. Statistical significances were analyzed using InStat 3 (GraphPad Software Inc., San Diego, USA). For comparison of at least two treated data groups with a control group, one-way analysis of variance (one-way ANOVA) was applied. For comparison between a treated group and a control group, Dunnett's post test was used with a level of error of $p < 0.05$ indicating significant and of $p < 0.01$ very significant differences. For direct comparison between two groups, the unpaired Student's t-test, Welch corrected, was used for calculation of a two-tail p value with $p = 0.05$–0.01 indicating significant, $p = 0.01$–0.001 very significant, and $p \leqslant 0.001$ highly significant differences.

3. Results

Here, we investigated the photo-(cyto)toxic, photo-mutagenic and photo-clastogenic potency of thirteen FC congeners and the coumarin limettin (Table 1) in V79 cells in culture. Besides the FCs previously tested for photo-toxicity and photo-mutagenicity, i.e., 5-MOP, 8-MOP, angelicin, bergamottin, bergaptol, DHB, isopimpinellin and psoralen (Raquet and Schrenk, 2009; Lohr et al., 2010; Messer et al., 2012), our analysis comprised a number of congeners with hitherto unknown or mostly unknown photo-toxic properties. These include byakangelicin, oxypeucedanin, oxypeucedanin hydrate, phellopterin, and sphondin. The determination of photo-(cyto)toxic (PTEF), photo-mutagenic (PMEF) and photo-clastogenic (PCEF) potency equivalency factors was carried out based on a concept of equivalency, relative to the potency of the most toxic congener, 5-MOP, analogous to the previously published equivalency concept for FC photo-mutagenicity (Raquet and Schrenk, 2009).

It was found that the proportionality requirements with respect to UVA dose and FC concentration for the calculation of equivalency factors (Raquet and Schrenk, 2009) were fulfilled for all compounds and endpoints tested (data not shown), 5-MOP being the most potent congener in all assays. It allowed the calculation of PTEF, PMEF and PCEF values for all test compounds, both on a mass basis and based on molarity in the assays (Table 1).

While most of the novel test compounds were either inactive or weakly photo-toxic, oxpeucedanin turned out to be a highly photo-(cyto)toxic congener. In comparison, its photo-mutagenic and photo-clastogenic potencies were weak to moderate. For most