Carcinogenesis vol.20 no.8 pp.1561–1566, 1999

Suppression of cell cycle progression by flavonoids: dependence on the aryl hydrocarbon receptor

John J.Reiners Jr[1], Russell Clift and Patricia Mathieu

Institute of Chemical Toxicology, Wayne State University, 2727 Second Avenue, Detroit, MI 48201, USA

[1]To whom correspondence should be addressed
Email: john.reiners.jr@wayne.edu

Some flavonoids are ligands of the aryl hydrocarbon receptor (AHR) and cause cell cycle arrest. The dependency of the cytostatic effects of five flavonoids (flavone, α-naphthoflavone, apigenin, 3′-methoxy-4'-nitroflavone and 2′-amino-3′-methoxyflavone) on a functional AHR was examined in AHR-containing rat hepatoma 5L cells and an AHR-deficient cell line (BP8) derived from the 5L line. The potent AHR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was cytostatic to the 5L line due to the induction of a G_1 arrest and dramatically elevated steady-state levels of CYP1A1 mRNA. TCDD affected neither the proliferation nor CYP1A1 mRNA contents of BP8 cells. With the exception of apigenin, the flavonoids under study induced G_1 arrest in both 5L and BP8 cells when used at concentrations at which they functioned as AHR agonists, but not antagonists. Apigenin-treated 5L and BP8 cultures primarily arrested in G_2/M. The AHR-containing murine hepatoma cell line 1c1c7 arrested following exposure to AHR agonist concentrations of flavone and α-naphthoflavone, but not TCDD. Unlike the G_1 arrest observed in 5L cultures, the latter two flavonoids caused principally G_2/M arrest in 1c1c7 cells. These studies demonstrate that the cytostatic activities of flavonoids do not require the AHR and the site of checkpoint arrest with a specific flavonoid can vary with cell type.

Introduction

The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Numerous members of this family have cytostatic (1), apoptotic (2,3), anti-inflammatory (4,5), anti-angiogenic (1) and estrogenic (6) activities. In addition, several flavonoids are potent modulators of both the expression and activities of specific cytochrome P450 genes/proteins (7–9). Because of these activities, flavonoids have attracted attention as possible chemoprotective or chemotherapeutic agents.

The cytostatic and apoptotic activities of the flavonoids have been attributed to their modulation of several biological processes. Flavonoids are potent inhibitors of processes involved in mitogen signaling or DNA synthesis, including mitogen-activated receptor tyrosine kinase activities (2,10,11), protein kinase C (10–12), mitogen-activated protein kinase kinase (MEK) (13) and topoisomerase II (1). A select group of flavonoids also up-regulate the expression of the cdk inhibitor $p21^{WAF1}$ (14) and/or inhibit cdk2/4 or cdc2 activities (14–16). These properties seemingly provide an explanation for the G_1/S and/or G_2/M arrests seen in some cell types following flavonoid exposure. However, other factors must influence the checkpoint at which cells arrest. For example, apigenin induces exclusively G_2/M arrest in human HL60 cells (14) but G_1/S and G_2/M arrest in human diploid fibroblasts (15).

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor (17,18). Numerous xenobiotics, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are ligands of the AHR. A variety of biological activities have been attributed to TCDD. One such activity is the induction of G_1 arrest in some cell types (19,20). Recent studies, involving analyses of cells of the rat hepatoma 5L lineage expressing different amounts of the AHR, demonstrated that TCDD induction of G_1 arrest requires a functional AHR (20).

Many flavonoids are AHR ligands (7,8,21). However, the biological activities invoked by their binding to the AHR are concentration dependent. At the lower end of the concentration range at which they bind to the AHR many flavonoids function as AHR antagonists (8,21,22). As antagonists they bind to the receptor and can compete with TCDD for binding, but do not transform the AHR into an active transcription factor. However, at higher concentrations the same flavonoids function as AHR agonists and duplicate many of the activities of TCDD, including transcriptional activation of the *CYP1A1* gene (8,21,22). We are unaware of any published comparison of concentrations at which selected flavonoids are cytostatic and function as AHR ligands. However, the concentrations at which numerous flavonoids have been reported to be cytostatic are well within the range defining flavonoid–AHR binding (1,7,8,21).

The current study was designed to determine whether the cytostatic effects of flavonoids could be attributed to their functioning as AHR ligands. As a test system we employed rat hepatoma 5L and BP8 cells, the latter being an AHR null variant of 5L cells which does not G_1 arrest following exposure to TCDD (20). As an ancillary system we also employed murine hepatoma 1c1c7 cells, a cell line that is well characterized with respect to its AHR content and responsiveness to TCDD and several flavonoids (22–24). The flavonoids examined (Figure 1) were chosen on the basis of existing literature documenting the concentrations at which the agents are cytostatic, function as AHR antagonists/agonists and/or inhibitors of MEK. Collectively, our studies demonstrate that the cytostatic properties of flavonoids are not dependent upon the presence of a functional AHR.

Materials and methods

Chemicals

Flavone was purchased from Aldrich Chemical Co. (Milwaukee, WI). Apigenin and α-naphthoflavone (α-NF) were obtained from Sigma Chemical Co.

Abbreviations: α-NF, α-naphthoflavone or 7,8-benzoflavone; AHR, aryl hydrocarbon receptor; DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; PBS, phosphate-buffered saline; PD98059, 2′-amino-3′-methoxyflavone; PD168641, 3′-methoxy-4'-nitroflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

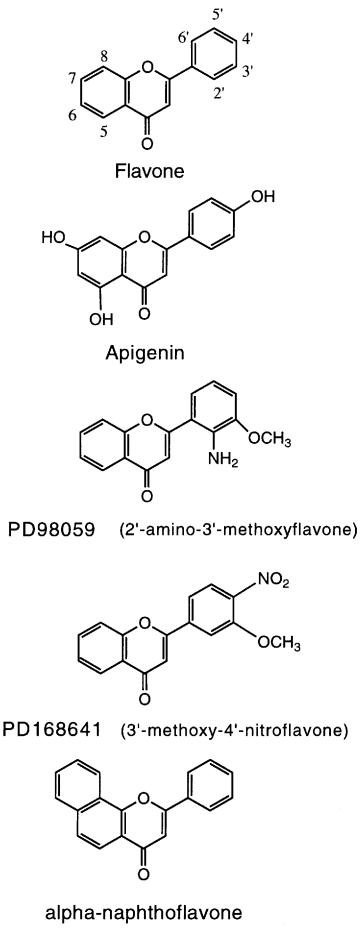

Fig. 1. Structures of flavone and related flavonoids.

(St Louis, MO). TCDD and 2′-amino-3′-methoxyflavone (PD98059) were purchased from ChemSyn (Lenexa, KS) and New England Biolabs (Beverley, MA), respectively. 3′-Methoxy-4'-nitroflavone (PD168641) was the gift of the Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co. (Ann Arbor, MI).

Cell culture and treatments

The rat hepatoma 5L and BP8 cell lines were gifts of Dr Martin Gottlicher (Research Center, Karlsruhe, Germany). Both lines were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and grown at 37°C in a humidified atmosphere containing 5% CO_2. For cell proliferation studies exponentially growing cultures were trypsinized and plated at low density in 60 mm dishes. Cultures were initially washed and refed 20–24 h after plating and then exposed to chemical agents ~24 h later. In a few studies the washing step was skipped and cultures were treated ~24 h after plating. The two protocols yielded similar results when examined in a side by side comparison.

Wild-type Hepa 1c1c7 cells (obtained from Dr J.Whitlock, Stanford University, CA) were cultured at 32°C as described previously (24).

Cultures were treated with varying concentrations of chemicals dissolved in dimethylsulfoxide (DMSO) (absolute volume of solvent <0.1% of medium volume). Details of treatment are provided in the text. Cells were harvested by exposure to solutions of 0.25% trypsin, 0.1 mM EDTA, counted and assessed for ability to exclude trypan blue.

RNA preparation and northern blot analyses

Total cellular RNA was isolated according to the acidic phenol extraction method of Chomeczynski and Sacchi (25). RNA was resolved on 1.2% agarose–formaldehyde gels and transferred to nylon membranes as described previously (13). The probes used for detection of 7S and rat and murine CYP1A1 RNAs and the conditions used for hybridization have been described in detail (24,26).

Cell cycle analyses

Cells were plated in 100 mm culture dishes at densities that ensured that they would still be in exponential growth at the time of harvest. Cultures were

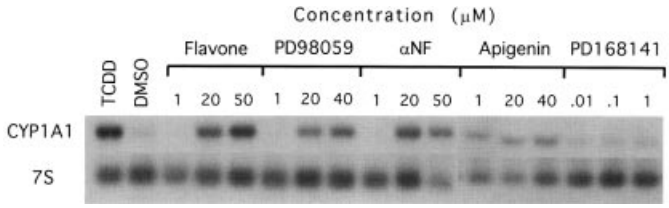

Fig. 2. CYP1A1 mRNA contents of 5L cells treated with flavonoids. Exponentially growing cultures of 5L cells were exposed to DMSO, TCDD (2 nM) or varying concentrations of flavonoids for 6 h prior to being harvested for isolation of RNA and analyses of CYP1A1 and 7S RNAs.

treated and harvested as described above. After trypsinization cells were washed once in phosphate-buffered saline (PBS), pelleted, and resuspended in PBS, which was followed by addition of an equal volume of ethanol. The resulting cell suspension was stored for a minimum of 3 days at 4°C before being processed for DNA analyses. For DNA analyses suspensions of ~1×10^6 cells were pelleted and resuspended in PBS containing glucose, 100 U/ml RNase A and 50 μg/ml propidium iodide. After a 2 h incubation in the dark the cells were analyzed with a Becton Dickinson FACScalibur instrument and the percentages of cells in the G_1, S and G_2/M stages of the cell cycle were determined with a DNA histogram-fitting program (MODFIT; Verity Software, Topsham, ME). A minimum of 10^4 events/sample was collected for subsequent analyses.

Results

Effects of TCDD and flavonoids on CYP1A1 mRNA steady-state content in 5L and BP8 cells

Exposure of 5L cultures to 1 nM TCDD resulted in a pronounced accumulation of CYP1A1 mRNA (Figure 2) within 6 h of exposure. Exposure to flavone, α-NF and PD98059 also caused concentration-dependent elevations of steady-state CYP1A1 mRNA contents in 5L cells (Figure 2). All of these flavonoids markedly increased CYP1A1 mRNA contents when used at concentrations ⩾20 μM, but had no detectable effects at 1 μM. CYP1A1 mRNA contents were also elevated in 5L cultures following exposure to apigenin, but to a lesser extent than that observed with flavone, α-NF and PD98059 (Figure 2).

The flavonoid PD168641 is a high affinity ligand of the AHR which functions exclusively as an AHR antagonist (7,21). Exposure of 5L cultures to concentrations of PD168641 as high as 1 μM had no effect on CYP1A1 mRNA content (Figure 2). This concentration is at least 50-fold greater than that needed to inhibit TCDD binding to the AHR and activation of *CYP1A1* (7,21).

Analyses identical to those presented in Figure 2 were also performed with the BP8 cell line. We were unable to detect either constitutive levels of CYP1A1 mRNA in this cell line or an accumulation of CYP1A1 mRNA following exposure to TCDD or any of the five flavonoids under study by northern blot analysis (J.J.Reiners, unpublished observations).

Cytostatic effects of TCDD and flavonoids in 5L and BP8 cells

Exposure of 5L cultures to concentrations of TCDD ⩾1 nM resulted in a transient suppression of proliferation (Figure 3). In contrast, 1 or 10 nM TCDD had no effect on BP8 proliferation (Figure 3). The AHR ligand and antagonist PD168641 had no effect on the proliferation of either 5L or BP8 cultures at even the highest concentration tested (e.g. 1 μM; Figure 3). A sub-agonist concentration (1 μM) of flavone, α-NF, apigenin or PD98059 did not affect the proliferation of either 5L or BP8 cultures (Figure 3). In contrast, all four flavonoids were cytostatic to both cell lines when used at concentrations (⩾20 μM) at which they functioned as AHR agonists (Figure 3). In general, 5L cells were as sensitive or

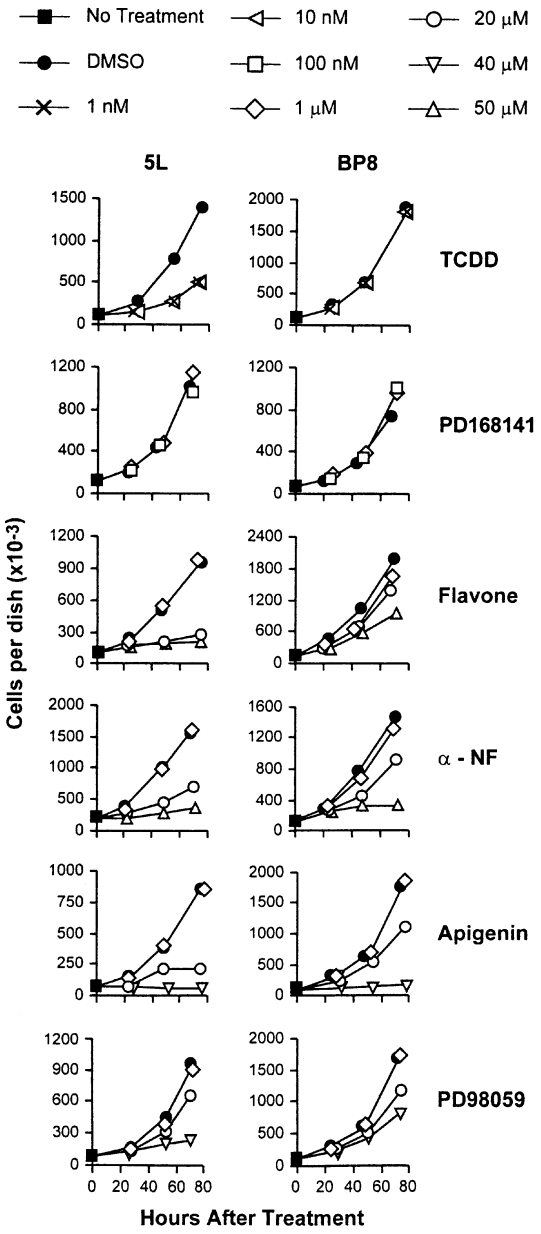

Fig. 3. Cytostatic effects of flavonoids on 5L and BP8 cells. Exponentially growing cultures of 5L and BP8 cells were exposed to DMSO, varied concentrations of TCDD or flavonoids prior to being harvested for determination of cell number. One set of untreated culture dishes was harvested at the time of treatment to serve as the 0 time control. Data represent means ± SD of determinations made on three to four culture dishes. All analyses were reproduced in a minimum of one additional study. Symbols are defined in the figure.

more sensitive than BP8 cells to the anti-proliferative effects of the flavonoids. Indeed, flavone concentrations ⩾20 μM completely suppressed the proliferation of 5L cells, but were only modestly cytostatic to BP8 cells.

In almost all cases examined, the anti-proliferative effects of the various flavonoids did not reflect agent cytotoxicity (J.J.Reiners, unpublished observations). There were only two notable exceptions. Exposure of 5L cultures to 50 μM flavone or 40 μM apigenin led to a time-dependent accumulation of trypan blue permeable cells (~40% after 72 h treatment). In contrast, 50 μM flavone was not cytotoxic to BP8 cells and 40 μM apigenin was only weakly cytotoxic (~15% killing after 72 h treatment).

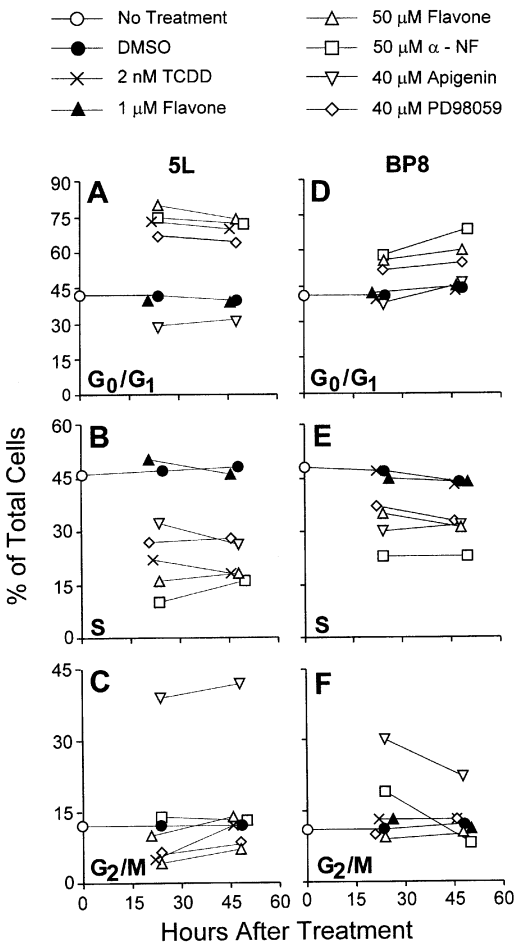

Fig. 4. Cell cycle distribution analyses of flavonoid-treated 5L and BP8 cells. Exponentially growing cultures of 5L and BP8 cells were exposed to DMSO, TCDD or 1, 40 or 50 μM concentrations of flavonoids prior to being harvested for per cell DNA analyses by FACs. Data represent analyses of 10^4 cells. Results similar to those depicted in the figure were obtained in a second independent study. Symbols are defined in the figure.

Cell cycle analyses of flavonoid-treated 5L and BP8 cells

Flow cytometric analyses of cellular DNA content were used to determine if the cytostatic effects of TCDD and the flavonoids reflected an arrest at a specific cell cycle checkpoint. TCDD treatment of 5L cells resulted in a G_1 arrest, as indicated by a dramatic increase in the percentage of cells having diploid DNA contents (Figure 4A), and concomitant reductions in the percentages of S and G_2/M phase cells (Figure 4B and C, respectively). In agreement with the proliferation data presented in Figure 3, TCDD had no effect on the cell cycle distribution of BP8 cells (Figure 4D–F). Flavone, α-NF and PD98059, like TCDD, also induced a pronounced G_1 arrest in 5L cultures when used at AHR agonist concentrations (40–50 μM; Figure 4A). A G_1 arrest also occurred in BP8 cultures treated with 50 μM flavone, 50 μM α-NF and 40 μM PD98059 (Figure 4D). However, in accord with the cell proliferation data reported in Figure 3, the percentage of BP8 cells in G_1 was less than that seen in 5L cultures treated with comparable concentrations of the flavonoids. Apigenin induced a dramatic accumulation of G_2/M cells in both 5L and BP8 cultures (Figure 4C and F).

Cell cycle analyses were also performed on 5L and BP8 cultures following exposure to a non-AHR agonist concentration (1 μM) of flavonoids. In no instance did 1 μM flavone,

α-NF, apigenin or PD98059 cause a perturbation of the cell cycle (1 μM flavone data are presented in Figure 4; other results are unpublished observations).

Effects of TCDD and flavonoids on CYP1A1 mRNA steady-state content in 1c1c7 cells

The murine hepatoma cell line 1c1c7 expresses relatively high levels of the AHR (27) and is very responsive to TCDD, as indicated by monitoring the transcriptional activation of *Cyp1a1* (Figure 5). CYP1A1 mRNA contents were also elevated in 1c1c7 cultures within 6 h of exposure to either 50 μM flavone or α-NF (Figure 5). A 1 μM concentration of either flavonoid had no effect on CYP1A1 mRNA steady-state content (Figure 5).

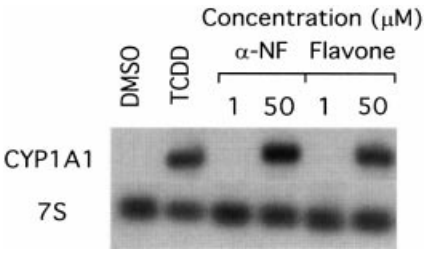

Fig. 5. CYP1A1 mRNA contents of 1c1c7 cells treated with flavonoids. Exponentially growing 1c1c7 cultures were exposed to DMSO, TCDD (5 nM) or varying concentrations of flavonoids for 6 h prior to being harvested for isolation of RNA and analyses of CYP1A1 and 7S RNAs.

Cytostatic effects of TCDD and flavonoids in 1c1c7 hepatoma cells

Exposure of 1c1c7 cultures to a concentration of TCDD capable of transcriptionally activating *Cyp1a1* had no effect on cell proliferation (Figure 6A), viability (Figure 6D) or cell cycle distribution (Figure 6G–I). In contrast, an AHR agonist concentration (50 μM) of flavone or α-NF totally suppressed the proliferation of 1c1c7 cells (Figure 6B and C). This suppression occurred in the absence of significant cytotoxicity (Figure 6E and F). In flavone-treated cultures the cells in G_1 initially arrested in G_1, whereas cells in S phase progressed into G_2/M and subsequently arrested (Figure 6G–I). However, within 72 h of treatment the flavone-induced G_1 arrest was lost and the cells progressed into the S and G_2/M phases of the cell cycle. In marked contrast to what was observed with flavone, no G_1 arrest occurred in 1c1c7 cultures following exposure to α-NF (Figure 6G). Instead, α-NF-treated cells underwent a sustained G_2/M arrest (Figure 6I).

Discussion

One goal of the current study was to determine if the cytostatic activities of flavonoids were related to their functioning as

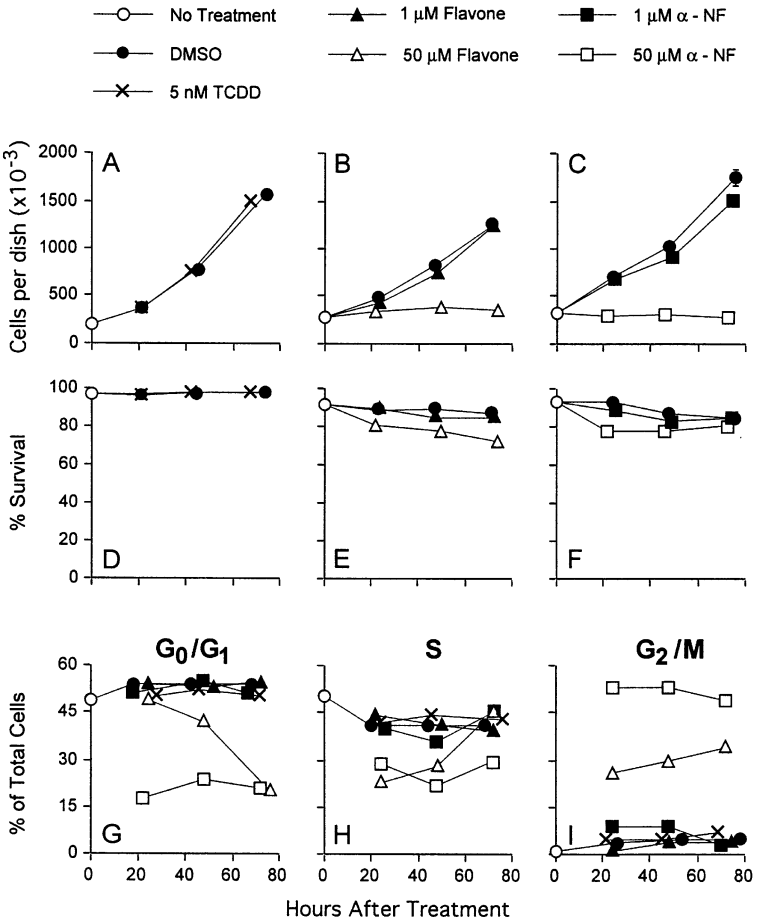

Fig. 6. Cytostatic and cytotoxic effects of flavonoids on 1c1c7 cells. Exponentially growing cultures were exposed to DMSO, TCDD (5 nM) or varied concentrations of flavonoids prior to being harvested for determination of cell number (**A–C**), viability (**D–F**) and DNA content (**G–I**). One set of untreated culture dishes was harvested at the time of treatment to serve as the 0 time control. Data represent means ± SD of determinations made on three to four culture dishes. All analyses were reproduced in a minimum of one additional study. Symbols are defined in the figure.

AHR ligands. Flavone, α-NF and TCDD were all capable of transforming the AHR of 1c1c7 cells into a functional transcription factor, as indicated by the accumulation of CYP1A1 mRNA. However, only the two flavonoids inhibited 1c1c7 cell proliferation. Since TCDD could transform the AHR into a functional transcription factor but did not duplicate the cytostatic effects of the flavonoids, it seems unlikely that the cytostatic effects of the flavonoids in this cell line reflected consequences of their interaction with the AHR. Similarly, although three of the flavonoids under study were cytostatic to 5L cells when used at concentrations at which they functioned as AHR agonists, and induced a G_1 arrest similar to the known AHR agonist TCDD, the same compounds also induced a G_1 arrest in AHR null BP8 cells. Furthermore, we recently found that pretreatment of 5L cultures with the AHR antagonist PD168641 suppressed the cytostatic activity of TCDD (J.J.Reiners, unpublished observation). However, a similar pretreatment of 5L cultures with PD168641 did not suppress the cytostatic effects of either apigenin or flavone. This latter study suggests that these two flavonoids suppress 5L proliferation by a process that does not involve the AHR. Collectively, these results demonstrate that the cytostatic activity of flavonoids is not dependent upon their interaction with the AHR.

Apigenin is cytostatic towards many cell types. However, the checkpoint at which cell cycle arrest occurs varies markedly amongst cell types. It induces predominantly a G_2/M arrest in human HL60 cells (15), B104 rat neuronal cells (28) and the immortal murine keratinocyte C50 cell line (15), but a mixed G_1 and G_2/M arrest in human diploid foreskin fibroblasts (14). HL60 cells express low levels of AHR mRNA and are not responsive to TCDD, as assessed by measurement of CYP1A1 mRNA (29). Similarly, the conditions used for the culturing of the C50 line suppress activation of *Ah* battery genes in primary cultures of murine keratinocytes by TCDD (30). Conversely, it has been reported that established fibroblast cell lines and primary fibroblast cultures constitutively express functional AHR (31,32). Although these studies may be interpreted as suggesting a correlation between AHR content/function and the checkpoint at which apigenin causes cell cycle arrest, our studies do not support such a correlation. Specifically, apigenin-induced a G_2/M arrest in both AHR null BP8 cells and the parental AHR-containing 5L cell line. Hence, the checkpoint at which apigenin arrests cells is not AHR-dependent.

PD98059 is a commonly used reagent in the signal transduction field because of the specificity with which it inhibits MEK (33,34). Studies employing PD98059 suggest that a functional MEK/extracellular signal-regulated kinase (ERK) pathway is required for mitogen-induced cell proliferation in several cell types (35–38). Reports by several investigators, including ourselves, have demonstrated that the phosphorylated, active forms of ERK1/2 are absent in a variety of cell types treated with concentrations of PD98059 $\geqslant$20 μM (13,33,34). Such concentrations suppressed the proliferation of both 5L and BP8 cells in the current study. Insufficient data are available to address the issue of whether flavonoids in general are MEK inhibitors. However, the current investigation, when coupled with our recent report (13), clearly demonstrates that the cytostatic activity of at least one flavonoid is unrelated to its ability to inhibit MEK/ERKs. Specifically, the concentration (20 μM) at which flavone was very cytostatic to 5L cultures is minimally 10-fold less than the ID_{50} required for MEK inhibition (13).

The cytostatic activity of TCDD has been documented in several cell types besides 5L cells. TCDD suppresses the proliferation of estrogen receptor-negative MDA-MD-486 human breast cancer cells (39) and estrogen receptor-positive MCF-7 human breast cancer cells (19,40). In the former case suppression is mediated by the production of a cytokine (i.e. transforming growth factor α), which inhibits proliferation in an autocrine fashion (39). In the latter case, the cytostatic effects of TCDD reflect its abilities to function as an anti-estrogen and down-regulate a battery of proliferative processes normally activated by 17β-estradiol (19). TCDD has also been reported to inhibit the proliferation of T47D human breast cancer cells (41) and sparsely seeded primary rat hepatocytes in response to epidermal growth factor (42). In this latter example, parallel studies performed with hepatocytes having a low affinity AHR implicated the AHR as being the mediator of the effects of TCDD. In contrast to these reports, our studies with 1c1c7 cells clearly demonstrate that TCDD is not necessarily cytostatic to cells having a functional AHR. We have also observed that concentrations of TCDD capable of transcriptionally activating several members of the *Ah* battery in the human breast epithelial cell line MCF10A do not affect the proliferation of such cells (43; unpublished observation). Hence, TCDD-dependent transformation of the AHR does not ensure the induction of a cytostatic process. Other factors, in concert with the AHR, must contribute to the susceptibility of a cell to the cytostatic effects of TCDD.

Acknowledgements

This work was supported by National Institutes of Health grant CA34469 and utilized the services of the Cell Culture and the Imaging and Cytometry Facility Cores, which are supported by National Institutes of Environmental Health Sciences grant P30ES06639.

References

1. Fotsis,T., Pepper,M.S, Aktas,E., Breit,S., Rasku,S., Adlercreutz,H., Wahala,K., Montesano,R. and Schweigerer,L. (1997) Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. *Cancer Res.*, **57**, 2916–2921.
2. Azuma,Y., Onishi,Y., Sato,Y. and Kizaki,H. (1995) Effects of protein tyrosine kinase inhibitors with different modes of action on topoisomerase activity and death of IL-2-dependent CTLL-2 cells. *J. Biochem. (Tokyo)*, **118**, 312–318.
3. Plaumann,B., Fritsche,M., Rimpler,H., Brandner,G. and Hess,R.D. (1996) Flavonoids activate wild-type p53. *Oncogene*, **13**, 1605–1614.
4. Ferrandiz,M.L. and Alcaraz,M.J. (1991) Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. *Agents Actions*, **32**, 283–288.
5. Onwukaeme,N.D. (1995) Anti-inflammatory activities of flavonoids of *Baphia nitida* Lodd. (Leguminosae) on mice and rats. *J. Ethnopharmacol.*, **46**, 121–124.
6. Breinholt,V. and Larson,J.C. (1998) Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay. *Chem. Res. Toxicol.*, **11**, 622–629.
7. Lu,Y.-F., Santostefano,M., Cunningham,B.D.M., Threadgill,M.D. and Safe,S. (1995) Identification of 3′-methoxy-4'-nitroflavone as a pure aryl hydrocarbon (Ah) receptor antagonist and evidence for more than one form of the nuclear Ah receptor in MCF-7 human breast cancer cells. *Arch. Biochem. Biophys.*, **316**, 470–477.
8. Lu,Y.-F., Santostefano,M., Cunningham,B.D.M., Threadgill,M.D. and Safe,S. (1996) Substituted flavones as aryl hydrocarbon (Ah) receptor agonists and antagonists. *Biochem. Pharmacol.*, **51**, 1077–1087.
9. Zhai,S., Dai,R., Friedman,F.K. and Vestal,R.E. (1998) Comparative inhibition of human cytochromes P450 1A1 and 1A2 by flavonoids. *Drug. Metab. Dispos.*, **26**, 989–992.
10. Agullo,G., Gamet-Payrastre,L., Manenti,S., Viala,C., Remesy,C., Chap,H.

and Payrastre,B. (1997) Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochem. Pharmacol.*, **53**, 1649–1657.
11. Hagiwara,M., Inoue,S., Tanaka,T., Nunoki,K., Ito,M. and Hidaka,H. (1988) Differential effects of flavonoids as inhibitors of tyrosine protein kinases and serine/threonine protein kinases. *Biochem. Pharmacol.*, **37**, 2987–2992.
12. Ferriola,P.C., Cody,V. and Middleton,E.Jr (1989) Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure–activity relationships. *Biochem. Pharmacol.*, **38**, 1617–1624.
13. Reiners,J.J.Jr, Lee,J.-Y., Clift,R.E., Dudley,D.T. and Myrand,S.P. (1998) PD98059 is an equipotent antagonist of the aryl hydrocarbon receptor and inhibitor of mitogen-activated protein kinase kinase. *Mol. Pharmacol.*, **53**, 438–445.
14. Lepley,D.M. and Pelling,J.C. (1997) Induction of p21/WAF1 and G_1 cell-cycle arrest by the chemopreventive agent apigenin. *Mol. Carcinog.*, **19**, 74–82.
15. Lepley,D.M., Li,B., Birt,D.F. and Pelling,J.C. (1996) The chemopreventive flavonoid apigenin induces G_2/M arrest in keratinocytes. *Carcinogenesis*, **17**, 2367–2375.
16. Carlson,B.A., Dubay,M.M., Sausville,E.A., Brizuela,L. and Worland,P.J. (1996) Flavopiridol induces G1 arrest with inhibition of cyclin-dependent kinase CDK2 and CDK4 in human breast carcinoma cells. *Cancer Res.*, **56**, 2973–2978.
17. Hankinson,O. (1995) The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.*, **35**, 307–340.
18. Schmidt,J.V. and Bradfield,C.A. (1996) Ah receptor signaling pathways. *Annu. Rev. Cell Dev.*, **12**, 55–89.
19. Wang,W., Smith,R. and Safe,S. (1998) Aryl hydrocarbon receptor-mediated antiestrogenicity in MCF-7 cells: modulation of hormone-induced cell cycle enzymes. *Arch. Biochem. Biophys*., **356**, 239–248.
20. Weiss,C., Kolluri,S.K., Kiefer,F. and Gottlicher,M. (1996) Complementation of Ah receptor deficiency in hepatoma cells: negative feedback regulation and cell cycle control by the Ah receptor. *Exp. Cell Res.*, **226**, 154–163.
21. Gasiewicz,T.A., Kende,A.S., Rucci,G., Whitney,B. and Willey,J.J. (1996) Analysis of structural requirements for Ah receptor antagonist activity: ellipticines, flavones, and related compounds. *Biochem. Pharmacol.*, **52**, 1787–1803.
22. Wilhelmsson,A., Whitelaw,M.L., Gustafsson,J.-A. and Poellinger,L. (1994) Agonistic and antagonistic effects of α-naphthoflavone on dioxin receptor function. *J. Biol. Chem.*, **269**, 19028–19033.
23. Merchant,M., Morrison,V., Santostefano,M. and Safe,S. (1992) Mechanism of action of aryl hydrocarbon receptor antagonist: inhibition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced *CYP1A1* gene expression. *Arch. Biochem. Biophys.*, **298**, 389–394.
24. Reiners,J.J.Jr, Scholler,A., Bischer,P., Cantu,A.R. and Pavone,A. (1993) Suppression of cytochrome P450 *Cyp1a-1* induction in murine hepatoma 1c1c7 cells by 12-*O*-tetradecanoylphorbol-13-acetate and inhibitors of proteins kinase C. *Arch. Biochem. Biophys.*, **301**, 449–454.
25. Chomezynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
26. Kocarek,T.A. and Reddy,A.B. (1996) Regulation of cytochrome P450 expression by inhibitors of hydroxymethyglutaryl-coenzyme A reductase in primary cultured rat hepatocytes and in rat liver. *Drug Metab. Dispos.*, **24**, 1197–1204.
27. Holmes,J.L. and Pollenz,R.S. (1997) Determination of aryl hydrocarbon receptor nuclear translocator protein concentration and subcellular localization in hepatic and nonhepatic cell culture lines: development of quantitative Western blotting protocols for calculation of aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein in total cell lysates. *Mol. Pharmacol.*, **52**, 202–211.
28. Sato,F., Matsukawa,Y., Matsumoto,K., Nishino,H. and Sakai,T. (1994) Apigenin induces morphological differentiation and G_2-M arrest in rat neuronal cells. *Biochem. Biophys. Res. Commun.*, **204**, 578–584.
29. Hayashi,S-i., Okabe-Kado,J., Honma,Y. and Kawajiri,K. (1995) Expression of Ah receptor (TCDD receptor) during human monocyte differentiation. *Carcinogenesis*, **16**, 1403–1409.
30. Jones,C.L. and Reiners,J.J.Jr (1997) Differentiation status of cultured murine keratinocytes modulates induction of genes responsive to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Arch. Biochem. Biophys.*, **347**, 163–173.
31. Zhang,L., Savas,U., Alexander,D.L. and Jefcoate,C.R. (1998) Characterization of the mouse Cyp1B1 gene. Identification of an enhancer region that directs aryl hydrocarbon receptor-mediated constitutive and induced expression. *J. Biol. Chem.*, **273**, 5174–5183.
32. Eltom,S.E., Larson,M.C. and Jefcoate,C.R. (1998) Expression of CYP1B1 but not CYP1A1 by primary cultured human mammary stromal fibroblasts constitutively in response to dioxin exposure: role of the Ah receptor. *Carcinogenesis*, **19**, 1437–1444.
33. Dudley,D.T., Pang,L., Decker,S.J., Bridges,A.J. and Saltiel,A.R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA*, **92**, 7686–7689.
34. Alessi,D.R., Cuenda,A., Cohen,P., Dudley,D.T. and Saltiel,A.R. (1995) PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*. *J. Biol. Chem.*, **270**, 27489–27494.
35. Kawata,Y., Mizukami,Y., Fujii,Z., Sakumura,T., Yoshida,K. and Matsuzaka,M. (1998) Applied pressure enhances cell proliferation through mitogen-activated protein kinase activation in mesangial cells. *J. Biol. Chem.*, **273**, 16905–16912.
36. Porras,A., Alvarez,A,M., Valladares,A. and Benito,M. (1998) p42/p44 mitogen-activated protein kinases activation is required for the insulin-like growth factor-I/insulin induced proliferation, but inhibits differentiation in rat fetal brown adipocytes. *Mol. Endocrinol.*, **12**, 825–834.
37. Coolican,S.A., Samuel,D.S., Ewton,D.Z., McWade,F.J. and Florini,J.R. (1997) The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J. Biol. Chem.*, **272**, 6653–6662.
38. Nelson,P.R., Yamamura,S., Mureebe,L., Itoh,H. and Kent,K.C. (1998) Smooth muscle cell migration and proliferation are mediated by distinct phases of activation of the intracellular messenger mitogen-activated protein kinase. *J. Vasc. Surg.*, **27**, 117–125.
39. Wang,W.L., Porter,W., Burghardt,R. and Safe,S.H. (1997) Mechanism of inhibition of MDA-MB-468 breast cancer cell growth by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Carcinogenesis*, **18**, 925–933.
40. Vogel,C. and Abel,J. (1995) Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on growth factor expression in the human breast cancer cell line MCF-7. *Arch. Toxicol.*, **69**, 259–265.
41. Fernandez,P. and Safe,S. (1992) Growth inhibitory and antimitogenic activity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in T47D human breast cancer cells. *Toxicol. Lett.*, **61**, 185–197.
42. Schrenk,D., Schafer,S. and Bock,K.W. (1994) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin as growth modulator in mouse hepatocytes with high and low affinity Ah receptor. *Carcinogenesis*, **15**, 27–31.
43. Reiners,J.J.Jr, Jones,C.L., Hong,N., Clift,R.E. and Elferink,C. (1997) Downregulation of aryl hydrocarbon receptor function and cytochrome P450 1A1 induction by expression of Ha-ras oncogenes. *Mol. Carcinog.*, **19**, 91–100.

Received November 19, 1998; revised March 12, 1999; accepted April 12, 1999