Identification of 3-methoxy-4-nitroflavone as a pure aryl hydrocarbon(Ah) receptor antagonist in MCF-7 human breast cancer cells

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Abstract:
3-Methoxy-4-nitroflavone competitively bound with high affinity to the rat cytosolic Ah receptor (TC_{50}=1.78 nM), but did not exhibit Ah receptor agonist activities in MCF-7 human breast cancer cells. In MCF-7 cells cotreated with 1 nM TCDD plus different concentrations of the flavone, there was a concentration-dependent inhibition of CYP1A1 gene expression and formation of the nuclear Ah receptor complex. Thus, 3-methoxy-4-nitroflavone is a pure Ah receptor antagonist in MCF-7 human breast cancer cells.

Introduction:
Bioflavonoids and related synthetic analogs exhibit a broad spectrum of biological activity. Several studies have reported that naturally-occuring polyhydroxylated flavonoids, chalcones and structurally related synthetic analogs exhibit antimutagenic and anticarcinogenic activities[1,2]. and inhibit activities of a number of enzymes including P450 isozymes[3]. The flavonoid-mediated effects are dependent on numerous factors including the structure of the compound, the target organ or cell and the response. The synthetic flavone, 5,6-benzoflavone(β-naphthoflavone) inhibited carcinogen-induced tumor formation in the mouse skin model and this activity was related, in part, to inhibition of P450-dependent metabolic activation of carcinogens[4,5]. Interestingly, β-naphthaflavone binds to the aryl hydrocarbon(Ah) receptor, whereas 7,8-benzoflavone(α-naphthaflavone, αNF) binds with moderate affinity to the Ah receptor and has been characterized as an Ah receptor antagonist[6-8]. Current studies in this laboratory have been investigating the modulatory effects of various 3,4-substituted flavones on Ah receptor-mediated signal transduction pathways and this report describes the activity and specificity of 3-methoxy-4-nitroflavone as an Ah receptor antagonist in MCF-7 human breast cancer cell line. This compound rapidly inactivates CYP1A1-dependent activity and inhibits TCDD-induced responses and the results obtained indicate that 3-methoxy-4-nitroflavone is a "pure" Ah receptor antagonist in MCF-7 cells.

Materials & Methods:
Chemicals and biochemicals: The 3,4-substituted flavone were synthesized as described[9]. TCDD, [^{3}H]TCDD(37 Ci/mmol), ethoxyresorufin and 2,3,7,8-tetrachlorodibenzofuran(TCDF) were synthesized in this laboratory to >98% purity. MCF-7 human breast cancer cells were originally obtained from ATCC. All other chemicals and biochemicals were of the highest purity available from commercial sources.

Preparation of hepatic cytosol: Rat hepatic cytosol was prepared according to the described procedure[10]

Hydroxyapatite(HAP) assay: The IC_{50} values for competitive receptor binding affinities were determined using freshly prepared rat hepatic cytosol (2 mg protein/ml) and the HAP procedure essentially as described[11].

Cytosolic AhR transformation assay: Rat hepatic cytosol was incubated with 5 nM TCDD in the presence or absence of different concentrations of flavone for 2 hr and AhR-DNA binding was

measured by gel mobility shift assay using a complementary oligonucleotide duplex containing the sequence 5'-GATCTGGCTCTTCTCACGCAACTCCG-3'[12].

Cell growth and EROD induction assay: MCF-7 cells were routinely grown in DME/F12 with 5% fetal calf serum. For the EROD induction assay, the cells were seeded into 35mm petri-dishes. TCDD and the compound dissolved in DMSO were added to the culture dishes when the cells reached about 70% confluency. Cells were harvested 24 hr after chemical treatment and EROD activity was determined by the method of Pohl and Fouts as previously described[13].

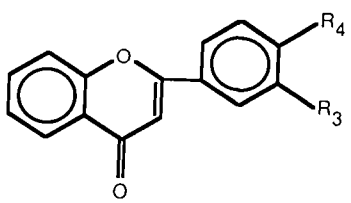
P4501A1 mRNA analysis: The CYP1A1 cDNA probe was purchased from ATCC. Total RNA from MCF-7 cells was isolated, electrophoresed, transferred to a nylon membrane, and probed with [^{32}P]-labeled CYP1A1 cDNA as described[7].

Preparation of nuclear extract: Near confluent MCF-7 cells were trypsinized and washed with the culture medium. Harvested cells were resuspended in 10 ml medium in 25-cm^2 culture flasks. [^{3}H] TCDD(10 nM) with or without the compound was added to the culture flasks. The cells were incubated by gentle shaking for 2 hr at 37°C. After incubation, the suspended cells were harvested and nuclear extracts were prepared as described[14].

Sucrose density gradient analysis of radiolabeled nuclear AhR: Aliquots of nuclear extract were layered onto linear sucrose gradient(5%~25%) and radiolabeled TCDD-AhR binding activity was analyzed as described previously[15].

Results & Discussion:

Previous studies have reported that several different structural classes of compounds including 6-methyl-1,3,8-trichlorodibenzofuran(MCDF) and related 6-substituted analogs and αNF inhibit diverse TCDD-induced responses in vivo and in mammalian cells in culture[16-17]. These compounds competitively bind to Ah receptor and block formation of the nuclear Ah receptor complex in various mammalian cells in culture[7]. Flavonoids exhibit a broad spectrum of activities and this report describes the characterization of 3-methoxy-4-nitroflavone as an Ah receptor antagonist (Fig.1). This compound was selected following an extensive prescreening of a series of synthetic 4- and 3,4-substituted flavones[9].



R_3- OCH_3

R_4- NO_2

Fig.1: Chemical structure of 3-methoxy-4-nitroflavone

Using rat hepatic cytosol, the concentration-dependent displacement of [^{3}H]TCDD by this compound was determined. The IC_{50} value was 2.27 ± 0.61 nM which is not significantly different from the value obtained for unlabeled TCDD (IC_{50}=1.78 nM) run in the same assay system. The corresponding K_i value was 1.46 ± 0.39 nM. Transformation of rat hepatic cytosolic Ah receptor by 3-methoxy-4-nitroflavone was also investigated using gel retardation assay and DRE binding. Compared to the DRE binding observed for 1 nM TCDD, 10 μM 3-methoxy-4-nitroflavone did not give detectable transformed DRE complex.

At concentrations from 0.1-10 μM, 3-methoxy-4-nitroflavone did not induce EROD activity in MCF-7 cells. In contrast, the EROD activity induced by 1 nM TCDD was 143.6 pmol/min/mg protein. The interactive effects were also investigated in MCF-7 cells treated with 1 nM TCDD plus different concentrations of the flavone (0.1-10 μM) for 24 h (Table 1). No significant inhibition of TCDD-induced EROD activity was observed in cells cotreated with 1 nM TCDD plus 0.1 μM of the flavone, whereas complete inhibition of TCDD-induced EROD activity was observed in cells cotreated with 1 nM TCDD plus 1 or 10 μM 3-methoxy-4-nitroflavone.

Table 1: Effect of 3-methoxy-4-nitroflavone on TCDD-induced EROD activity in MCF-7 cells

Treatment TCDD (nM)	Compound (μM)	EROD Activity (pmol/min/mg protein) (mean ± SD)
1	0	143.6 ± 9.8
1	0.1	124.1 ± 23.4
1	1	5.8 ± 6.4
1	10	5.7 ± 6.8
0	0	0

The activity of 3-methoxy-4-nitroflavone as an inducer of CYP1A1 gene expression was determined in MCF-7 cells by Northern blot analysis. The results illustrated in Fig.2 indicate that the flavone alone is inactive as an inducer at concentration as high as 10 μM, whereas 3-methoxy-4-nitroflavone significantly inhibited TCDD-induced CYP1A1 mRNA levels (lanes 9 and 10).

Fig. 2: Effect of 3-methoxy-4-nitroflavone as inducer of CYP1A1 mRNA levels in the presence or absence of 1 nM TCDD in MCF-7 cells. Total RNA was extracted from MCF-7 cells 24 hr after treatment with 0.01, 0.1, 1.0, and 10 μM 3-methoxy-4-nitroflavone, DMSO, 1 nM TCDD, 1 nM TCDD+0.01, 0.1, 1.0, and 10 μM 3-methoxy-4-nitroflavone (lanes 1 through 10, respectively). Northern analysis was carried out as described in the Materials & Methods section.

The effects of 3-methoxy-4-nitroflavone on formation of the nuclear Ah receptor complex were also determined in MCF-7 cells treated with 10 nM [^{3}H]TCDD and 1 or 10 μM of the flavone. At both concentrations, this flavone inhibited formation of the nuclear Ah receptor complex (Fig.3) in MCF-7 cells. However, an early-eluting specifically-bound peak which constituted approximately 10% of the total specifically-bound nuclear Ah receptor complex was not affected by this compound. The failure of this compound to inhibit formation of the early-eluting specifically-bound nuclear Ah receptor complex suggests that [^{3}H]TCDD interacts with more than one binding site in the Ah receptor or that there is more than one form of the Ah receptor.

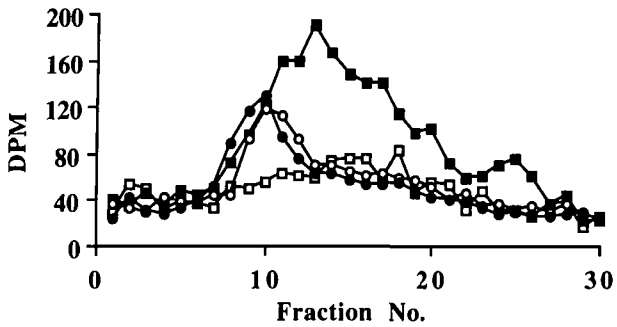


Fig.3: Velocity sedimentation analysis of nuclear extracts from MCF-7 cells treated with 10 nM [^{3}H]TCDD in the absence or presence of 1 or 10 μM unlabeled 3-methoxy-4-nitroflavone. MCF-7 cells were treated with [^{3}H]TCDD and different concentrations of compound, nuclear extracts were obtained and analyzed by sucrose density gradient centrifugation as described in Materials & Methods section. Levels of specifically-bound radiolabeled nuclear Ah receptor complex in cells treated with 10 nM [^{3}H]TCDD (■), 10 nM [^{3}H]TCDD plus 1 μM 3-methoxy-4-nitroflavone(○), 10 nM [^{3}H]TCDD plus 10 μM 3-methoxy-4-nitroflavone(●). Nonspecifically bound radioactivity was obtained from cells treated with 10 nM [^{3}H]TCDD and a 200-fold excess of unlabeled TCDF(□).

In summary, the results in this study demonstrate that 3-methoxy-4-nitroflavone is a pure Ah receptor antagonist and partially inhibits formation of the nuclear Ah receptor complex. This compound will be useful in the future as probe to further characterize the Ah receptor complex. (Supported by the National Institutes of Health, ES03843)

References:

1. Mitscher L.A., Telikepalli H., Wang P.B.B., Kuo S., Shankel D.M. and Stewart G.(1992) Mutat. Res. 267, 229-241.
2. Edenharder R., Von Petersdorff I. and Rauscher R. (1993) Mutat. Res. 287, 261-274.
3. Ertan R., Goker H., Ertan M., Beretz A., Cazenave J.P., Haag M. and Anton R. (1991) Chimie 26, 735-738.
4. Le Bon A.M., Siess M-H. ans Suschetet M. (1992) Chem.-Biol. Interact. 83, 65-71.
5. Nakadate T., Yamamoto S., Aizu E. and Kato R.(1984) Gann 75, 214-222.
6. Gasiewicz T.A. and Rucci G. (1991) Mol. Pharmacol. 40, 607-612.
7. Merchant M., Morrison V., Santostefano M. and Safe S. (1992) Arch. Biochem. Biophys. 298, 389-394.
8. Merchant M., Arellano L. ans Safe S. (1990) Arch. Biochem. Biophys. 281, 84-89.
9. Lesca P., Rafidinarivo P., Lecointe P. and Mansuy D. (1979) Chem.-Biol. Interact. 24, 189-198.
10. Santostefano M., Merchant M., Arellano L., Morrison V. Denison M.S, and Safe S. (1993) Mol. Pharmacol. 43, 200-206.
11. Gasiewicz,T.A. and Neal R.A.(1982) Anal. Biochem. 124, 1-11.
12. Denison M.S. and Deal R.M. (1990) Mol. Cell. Endocrinol. 69, 51-57.
13. Pohl R.J. and Fouts J.R. (1980) Anal. Biochem. 107, 150-155.
14. Wang X., Santostefano M., Lu Y. and Safe S. (1992) Chem.-Biol. Interact. 85, 79-93.
15. Harris M., Zacharewski T. and Safe S.(1990) Cancer Res. 50, 3579-3584.
16. Harris M., Zacharewski T., Astroff B. and Safe S.(1989) Mol. Pharmacol. 35, 729-735.
17. Astroff B. and Safe S. (1989) Toxicol. 59, 285-296.