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Evidence for Estrogenic Contamination of the MAPK Inhibitor PD98059

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ABSRACT: PD98059 blocks phosphorylation and activation of MAPK proteins, ERK1 and ERK2. In the course of examining the effect of PD98059 on estrogen-induced transcription of reporter genes in a human breast cancer cell line and in yeast, we found that two of four different batches of PD98059 produced estrogenic effects in a dose-dependent manner. In a competitive binding assay, these preparations of PD98059 displaced radiolabeled estradiol from ERα. Furthermore, in the yeast assay, addition of a coactivator protein, AIB1, enhanced the transcriptional effect of PD98059, indicating that it induces receptor-coactivator interactions. Although concentrations of PD98059 required to activate ERα in these experimental systems are 10^4- to 10^5 higher than the concentration of estradiol required to do the same, the concentrations required to block MAPK activation are well above those which would produce maximal estrogenic effects. Thus, when PD98059 is used in estrogen-responsive cells, contaminating estrogenic activity may confound interpretation of experimental results.

Introduction

Peptide growth factors can induce typical estrogenic responses (1). Mitogen activated protein kinase (MAPK) mediates cross-talk between growth factor and estrogen receptor (ER) pathways (2, 3). Activation of the MAPK proteins ERK1 and ERK2 requires their phosphorylation by an upstream kinase, MEK (4). PD98059 is a specific inhibitor of MEK and it has been used to study cross-talk between growth factor pathways and ER mediated events (5-7).

In the present study, we found that some batches of PD98059 exhibit estrogenic activity. Since PD98059 is a flavone compound, we compared its effects to the related compounds, apigenin and genistein. All three compounds displaced radiolabeled estradiol from receptor, stimulated ERα-coactivator interactions and enhanced transcription of ERE containing reporter constructs. These unexpected findings strongly suggest that contaminating estrogenic activity in some PD98059 preparations can greatly confound and perhaps even compromise interpretation of results of experiments using this compound on estrogen-responsive cells.

Materials and Methods

Chemicals

Four different lots of PD98059 (2'-amino-3'-methoxyflavone) (PD) were purchased from three sources: PD lot no. B24784 (PD-B24) and lot no. B30843 (PD-B30), Calbiochem-Novabiochem Corporation (La Jolla, CA); PD lot no. 80K4702 (PD-80K), Sigma Chemical Co. (St. Louis, MO); and PD lot no. P53549 (PD-P53), Biomol (Plymouth Meeting, PA). Estradiol (E2) was purchased from Sigma Chemical Co.

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Mammalian Estrogen Reporter Gene Assays

The ER-negative breast cancer cell line, MDA-MD-231, was maintained in EMEM containing phenol red and supplemented with 5%(v/v) fetal calf serum (FCS) and NEAA, glutamine, Hepes buffer, and 6ng/ml insulin (complete medium). For transfection, cells were seeded in 12-well plates in phenol-red free EMEM medium supplemented with 3% (v/v) charcoal-stripped serum plus NEAA, glutamine, and Hepes buffer (basal medium). After two days, cells were transfected using Lipofectamine (Life Technologies, Inc., Rockville, MD). Each culture well was transfected with expression vectors for: ERα (HEGO, from P. Chambon, Strasbourg, FR); an estrogen-responsive reporter construct (ERE2pS2-luc) and a control reporter construct, pCMV-β-galactosidase (ß-gal, Tropix, Foster City, CA), to determine transfection efficiency. ERE2pS2-luc was made by synthesizing the minimal promoter region of the pS2 gene, nucleotides –91 to +10 (8), and ligating this into the pGL3, luciferase reporter gene (Promega, Madison, WI). A clone containing the pS2-luc promoter in the correct orientation was further modified to make it estrogen responsive. A double stranded oligonucleotide containing two consensus ERE sites (underlined) was made with the following sequence: GTACCAGGTCACAGTGACCTGATCAGCTA-GTCAGGTCACAGTGACCTTCGTAC. This sequence was ligated into pS2-luc to make ERE2pS2-luc.

At 5 h after transfection, cells were treated with test compound. After an additional 20 h, cells were harvested and extracts were assayed for luciferase (Luciferase Assay System, Promega) and β-gal activities (Galacto-Light, Tropix). Results were expressed as the ratio luciferase:β-gal to correct for differences in transfection efficiency.

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ERα Binding Assay

Competition experiments were performed by incubating recombinant ERα (PanVera, Madison, WI) in solution with radiolabeled E_2 and separating receptor bound from free E_2 using the standard hydroxylapatite method. Stock ERα was diluted in a 10 mM tris-EDTA buffer containing 1 mM DTT (TED) and 1 mg/ml bovine serum albumin (fatty acid free BSA, Sigma). ERα (10 ng) was added to each reaction tube along with 1.5 nM $[^3H]E_2$ (Amersham, Arlington, IL) plus unlabeled competitor (E_2, PD98059) at various concentrations and incubated for 2 h at 4°C. Receptor bound radioligand was pulled out of solution by addition of 50% slurry of hydroxylapatite in TED buffer. The hydroxlyapatite was washed 4 times in TED at 4°C and then extracted with ethanol. The radioactivity in the ethanol supernatant was determined by liquid scintillation.

Yeast Estrogen Reporter Assay

Rat coactivator AIB1 (rAIB1) was cloned from a rat uterine cDNA library using a yeast two-hybrid cloning system (HybridZAP Two-Hybrid cDNA Gigapack Cloning Kit, Stratagene, La Jolla, CA). The carboxy-terminal portion of ERα (nucleotides 944 - 1876, Genbank accession no. X61098) was amplified by PCR and ligated, in frame, into the Gal4 DNA binding domain vector, pDB-Gal4, to make the hybrid pDB-Gal4-$ER\alpha_{291-600}$. Yeast cells (strain PJ69-4A, a gift from Dr. Philip James, Univ Wisconsin, Madison) were transformed with the uterine cDNA pAD-Gal4 plasmid library and hybrid pDB-Gal4-$ER\alpha_{291-600}$ using the lithium acetate method. Clones were selected by growth on SD medium (-His, - ADE) containing 10^{-8} M E_2. Clones that were positive for ß-gal expression under E_2 stimulation were further analyzed. The captured cDNA of these clones was sequenced. One clone contained rAIB1 coding sequence (Genbank accession no. AF322224), and this pAD-Gal4-rAIB1 was used in studies of ligand-induced, coactivator-ERα interactions.

The yeast estrogen-responsive reporter assay was performed as described previously (9). Briefly, yeast cells (RS188N) were transformed with vectors for ERα, the ERE-ß-gal reporter gene and either empty pAD-Gal4 vector or pAD-Gal4-rAIB1. Cells were grown overnight in selection medium (lacking uracil, tryptophan, and/or leucine) and then divided into treatment groups. Test compounds were added in DMSO. The ß-gal activity in the yeast was assayed 18 h later using a Galacto-Star luminescence assay kit (Tropix).

Inhibition of MEK Activity

The ER-positive breast cancer cell line, MCF-7, was maintained in complete medium. To test MEK activity, cells were seeded into 60 mm culture dishes and grown in basal medium until they were approximately 75% confluent. Serum-free medium was added to the cultures for 18 h and the cells were challenged with 10 ng/ml TGFα alone or in the presence of the different preparations of PD98059 at 1, 10, 50, or 100 μM. After 5 mins. the cells were lysed and the lysate was analyzed by immunoblot using an antibody specific for the phosphorylated form of MAPK (Cell Signaling Technology, Beverly, MA).

MEK activity was also monitored in a reporter gene transfection system, PathDetect (Stratagene, La Jolla, CA). In this system cells are transfected with an expression vector for a fusion protein containing the activation domain of the transcription factor ELK and the GAL4 DNA binding domain (pFA2-Elk1). The cells are also transfected with a luciferase reporter gene containing a GAL4 UAS upstream of the TATA box (pFR-Luc). The ELK fusion protein is activated upon phosphorylation by MAPK, binds to the GAL UAS and stimulates transcription of the reporter. MDA-MB-231 cells were transfected with pFA2-Elk1, pFR-Luc, and pCMV-ß-gal. After transfection, cells were treated for 20 hours with 10 ng/ml TGFα, in the presence of either estrogenic or non-estorgenic PD98059 at 0 – 100 μM. Luciferase activity was corrected for transfection efficiency by expressing it as the ratio of luciferase to ß-galactosidase activities.

Statistics

Results of gene reporter assays were analyzed by ANOVA, followed by Dunnett's test to compare individual means against the control mean.

Results & Discussion

In the course of experiments examining the role of MAPK on estrogen receptor activity, we found that PD98059 stimulated transcription of estrogen responsive reporter genes. Since there were no other reports of inherent estrogenic activity in PD98059, we tested the possibility that this effect was specific to this single batch of com-

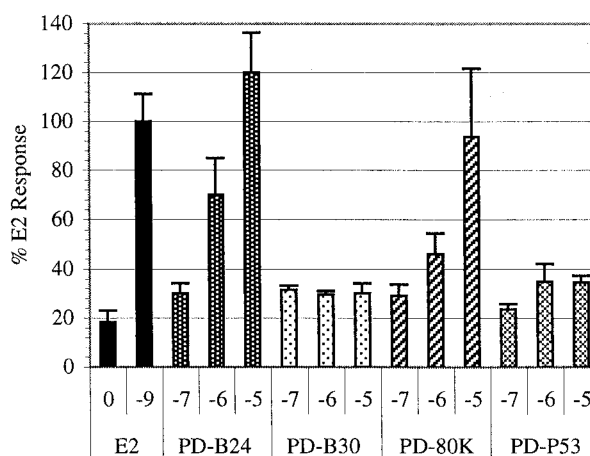

Fig. 1 Induction of an estrogen-responsive reporter gene in breast cancer cells. MDA-MB-231 cells were transfected with HEGO (ERα) and ERE2pS2-luc, and treated with E_2 or different batches of PD98059 (PD-#) at the indicated log dose. Reporter expression in the E2-treated group was set at 100% and all others were calculated relative to this level. Values are means ± SD, n = 4.

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pound, that perhaps the preparation was contaminated during manufacture. Indeed, when four different batches of PD98059 were tested, two produced a dose-dependent activation of the estrogen-responsive reporter gene in human breast cancer cells (Fig. 1). Furthermore, two bottles of PD-B24 that had been reconstituted on separate occasions three months apart, exhibited equivalent estrogenic potency (data not shown). The maximum effect of the active PD98059 preparations occurred at a nominal concentration of 10 μM; the concentration of PD98059 routinely used to inhibit MAPK activation in culture is 10-50 μM (5-7). Since PD98059 is a flavone compound, we compared it against apigenin and genistein, other flavanoids known to behave as estrogens (10). The potency of the active PD98059 preparations appeared equivalent to apigenin and approximately one-tenth that of the isoflavone, genistein (data not shown).

When tested in a receptor binding assay, the estrogenic batch, PD-B24 competed with radiolabeled estradiol, however, it required a concentration that was about 40,000-fold higher than estradiol to produce 50% displacement (Fig. 2). Also, the affinity of PD-B24 for estrogen receptor was approximately one-tenth of that exhibited by apigenin (data not shown). PD-80K also displaced estradiol from the receptor but at a much reduced rate; thus, the lower potency of this batch in the ERE-reporter assay is a reflection of its lower apparent affinity for the ERα. PD-B30 and PD-53 were without receptor binding activity, reflecting the lack of activity seen for these batches in the reporter assay. Since the MEK inhibitory action of PD98059 is usually achieved at 10-50 μM, concentrations that are 100,000-500,000 times higher than the EC_{50} for E_2-induced responses, the low level of ER binding activity in PD preparations would be significant under typical experimental conditions.

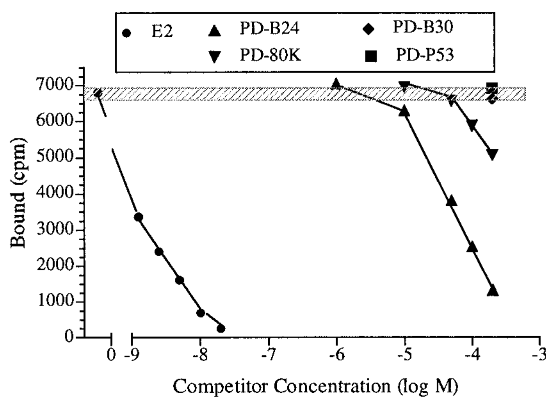

Fig. 2. Competitive binding for ERα. Recombinant ERα was incubated with 1.5 nM $[^3H]E_2$ and the indicated concentrations of competitor. Values are the average of replicate samples.

In the yeast estrogen reporter assay, the effect of treatment with PD-B24 was tested with or without the addition of rAIB1 (Fig. 3). Steroid receptors enhance gene expression by forming a complex with the transcriptional machinery through a bridge formed by coactivator proteins (11). The response of estrogen reporter genes to weakly estrogenic compounds is greatly enhanced in yeast by the addition of a mammalian coactivator protein (9, 12). In the absence of AIB1, PD-B24 had only a slight stimulatory effect at 10^{-5}M (Fig. 3A). When AIB1 was present, the potency of estradiol was increased 10-fold and PD-B24 was fully effective (Fig. 3B).

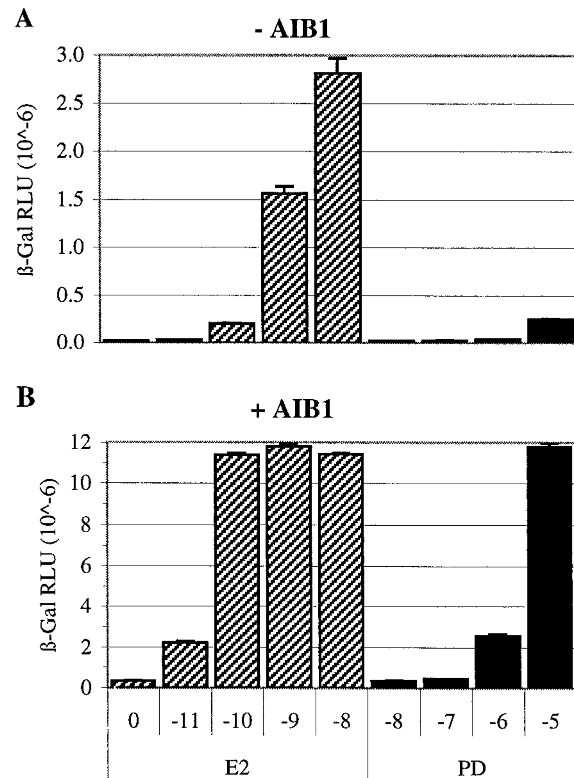

Fig. 3 Yeast estrogen reporter assay. Yeast cells were transformed with expression vectors for ERα and an estrogen-responsive reporter construct (ß-gal), in the absence (A) or presence (B) of the expression vector for rAIB1. Cells were treated with the indicated log concentration of compound (E_2 or PD-B24). Means ± SD (n=3).

The estrogenic contaminant does not affect the ability of the PD98059 to inhibit MEK. When MCF-7 cells were stimulated with TGFα, both estrogenic and non-estrogenic batches of PD98059 inhibited the production of phosphorylated MAPK and blocked ELK activation with equivalent potency (Fig. 4). Furthermore, this experiment demonstrates that full inhibition of MEK is only achieved when the concentration of PD98059 is at 50 μM or above, concentrations that would produce maximal estrogenic effects if the contaminated batches were used.

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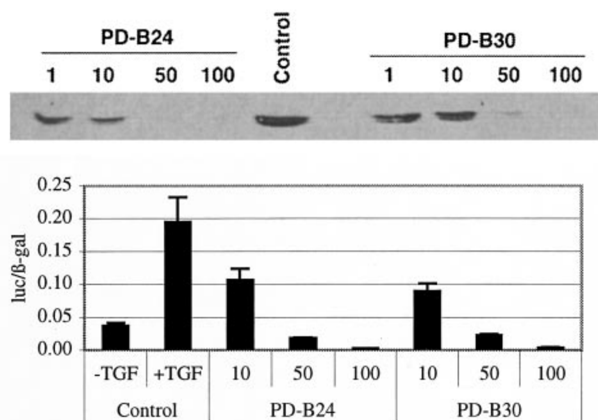

Fig. 4. MEK inhibition by estrogenic and non–estrogenic batches of PD98059. Upper Panel: MCF-7 cells were stimulated with TGFα alone (control) or in the presence of the indicated concentration (μM) of the two batches of PD98059 (PD-B24, PD-B30). Cells were lysed after 5 minutes and the amount of phosphorylated MAPK was detected in an immunoblot. Lower Panel: Activation of the transcription factor ELK was monitored using the PathDetect Trans-Reporter system. MDA-MB-231 cells were transfected and treated with TGFα alone (control) or in the presence of the indicated concentration (μM) of the two batches of PD98059. Values are the means ± SD of 4 replicates.

Whether the estrogenic contaminant of a PD98059 preparation produces an identifiable effect may depend on the test system under study. Certainly, in cases where a complex promoter is being tested, one with multiple response elements in addition to an estrogen response element, one might expect that the MAPK inhibitory effect could override any estrogenic effect. Such might be the case with the prolactin gene. Although there are EREs in the promoter region of the PRL gene, these regulatory elements do not act in isolation, *i.e.,* other *cis*-elements are required for the action of estrogen (13). MEK inhibition might predominate because the transcription factors for these other *cis*-elements are not activated, thereby ablating the response to any estrogenic stimuli.

In summary, different batches of the flavone compound PD98059 are estrogenic when tested in transcription enhancement assays involving simple promoters designed to be primarily responsive to estrogen action. This estrogenic activity stems from the ability of these preparations of PD98059 to bind and activate ERα, leading to an interaction of the receptor with cis-acting response elements and trans-acting coactivator proteins. The commercial source of inhibitor does not predict which batches will be estrogenic; indeed, one of two lots of the compound from the same source was estrogenic. The inherent estrogenic activity within certain preparations of PD98059 is likely to confound interpretation of experiments that involve an estrogen responsive system.

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