Interference by Naturally Occurring Fatty Acids in a Noncellular Enzyme-Based Aromatase Bioassay

Marcy J. Balunas,[†,‡] Bin Su,[‡] Serena Landini,[‡] Robert W. Brueggemeier,[‡] and A. Douglas Kinghorn*[,‡]

Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612, and Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210

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Natural product drug discovery efforts frequently utilize noncellular screening assays. Fatty acids are commonly found in natural product extracts, and some have been shown to interfere with noncellular assays. Several pure fatty acids were tested using a noncellular aromatase assay, with the unsaturated analogues showing strong inhibitory activity, while the saturated analogues were inactive. Unsaturated fatty acids were further tested against SK-BR-3 hormone-independent human breast cancer cells that overexpress aromatase and were found to be inactive. In natural product screening efforts, especially using plant seeds, it is recommended that extracts active in noncellular bioassays should be dereplicated for the presence of fatty acids prior to bioassay-guided fractionation.

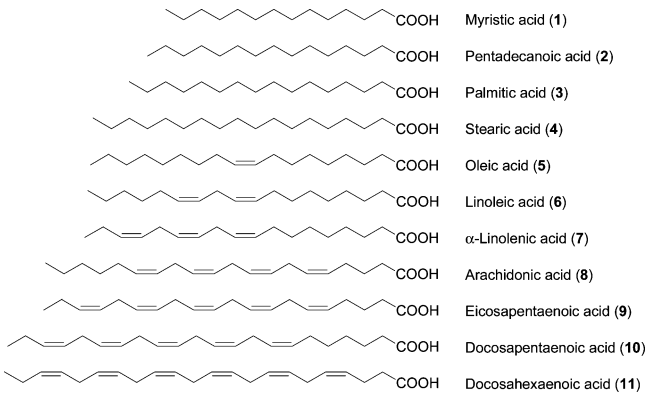
High-throughput screening (HTS) assays have become the dominant method for initial lead generation in the drug discovery process. HTS assays utilize noncellular and cell-based formats, both of which have benefits and drawbacks. Although cell-based assays allow for examination of results in a more relevant biological context, noncellular assays are often employed to investigate the inhibition, activation, or modulation of enzymes or receptors of biological interest. Natural product drug discovery efforts frequently utilize high-throughput noncellular and cell-based screening assays that test for various biological activities.[1,2]

The utilization of noncellular assays has proven to be an effective tool in natural product drug discovery. However, certain classes of compounds commonly found in natural product extracts have been shown to provide positive results in many different noncellular assays and are often considered "nuisance" compounds during bioassay-guided fractionation. For example, tannins are frequently found in numerous plant species from a wide range of plant families.[3,4] Although tannins are currently under investigation for their possible medicinal effects on various diseases,[4] these complex plant polyphenols have been shown to interact with numerous enzyme-based assays.[3] Because of the widespread interactions of tannins with noncellular assays, in many plant natural product drug discovery efforts an attempt is made to remove tannins prior to screening with noncellular assays, unless the isolation of tannins is of specific interest to the research program.

Fatty acids are another class of compounds that are widely distributed in natural product extracts. Except for archaebacteria, they are found in all organisms as components of cell membranes and products of metabolic processes[5] and are frequently present in natural product extracts. This class of natural products is especially common in plant seed extracts and can also be found in extracts of other plant parts, as well as in marine and microbial extracts. Various fatty acid analogues have been shown to provide positive results in initial screening of natural products with some noncellular assays, including COX-1/COX-2 enzyme inhibition,[6−9] adenosine A_1 and opiate receptor binding,[10] estrogen receptor binding,[11] collagenase and elastase inhibition,[12] and 5α-reductase inhibition assays.[13]

As part of a research program to find new aromatase inhibitors, the widespread presence of fatty acids in plant extracts prompted an investigation of the potential for these compounds to interfere with a noncellular aromatase assay.[14,15] Aromatase is the rate-limiting enzyme responsible for catalyzing the final step in the biosynthesis of estrogens (estrone and estradiol) from androgens (androstenedione and testosterone).[16,17] Inhibition of aromatase has been shown to reduce estrogen production throughout the body to nearly undetectable levels and is proving to have a significant effect on the development and progression of hormone-responsive breast cancers. The current study sought to determine if naturally occurring fatty acids could interfere with natural product screening results from a noncellular microsomal radiometric aromatase assay.

Several pure fatty acids (compounds **1**−**11**) were tested initially



at our standard screening concentration of 20 μg/mL in a noncellular microsomal radiometric aromatase assay,[14,15] including saturated and unsaturated fatty acids with a range of chain lengths (14−22 carbons), various numbers of double bonds (0−6), and varying positions of the first double bond from the methyl end of the compound (ω-3, ω-6, and ω-9). The assay measured the amount of tritiated water (^3H_2O) released as aromatase converts [1β-^{3}H]-androst-4-ene-3,17-dione to estrone. Saturated fatty acids [**1**, 66.7 percent control activity (PCA); **2**, 76.2 PCA; **3**, 83.2 PCA; and **4**, 89.4 PCA] showed no significant inhibitory activity in the noncellular aromatase assay (Figure 1, Table 1). In contrast, most of the unsaturated fatty acids were found to be active, including **8** (11.5 PCA), **11** (12.4 PCA), **10** (15.7 PCA), **5** (19.5 PCA), **6** (22.5 PCA), and **9** (30.2 PCA) [**7** was less active (49.5 PCA)] (Figure 1, Table 1). During large-scale screening efforts, extracts containing unsaturated fatty acids would show high levels of aromatase inhibition using this noncellular aromatase assay.

* To whom correspondence should be addressed. Tel: +1-614-247-8094. Fax: +1-614-247-8642. E-mail: kinghorn.4@osu.edu.
† University of Illinois at Chicago.
‡ The Ohio State University.

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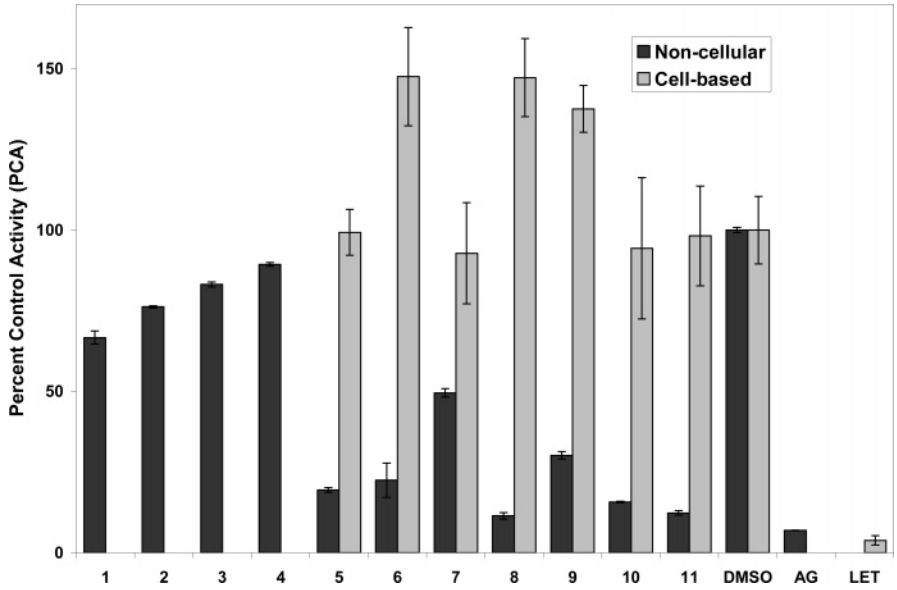

Figure 1. Percent control activity (PCA) for saturated and unsaturated fatty acids in the noncellular, enzyme-based aromatase bioassay and for unsaturated fatty acids in SK-BR-3 hormone-independent human breast cancer cells that overexpress aromatase (DMSO = dimethyl sulfoxide, blank/negative control; AG = aminoglutethimide, positive control in noncellular assay; LET = letrozole, positive control in cell-based assay).

Table 1. Percent Control Activity (PCA) for the Noncellular, Enzyme-Based and the SK-BR-3 Cell-Based Aromatase Bioassays and IC_{50} Values for the Noncellular, Enzyme-Based Bioassay

				noncellular bioassay			cell-based bioassay	
compound	chain length	double bonds	ω	PCA (20 μg/mL)	standard error	IC_{50} (μM)	PCA (100 μM)	standard error
1	14	0		66.7	2.06			
2	15	0		76.2	0.38			
3	16	0		83.2	0.77			
4	18	0		89.4	0.59			
5	18	1	9	19.5	0.74	32.7	99.3	7.10
6	18	2	6	22.5	5.31	48.0	147.6	15.21
7	18	3	3	49.5	1.30	44.2	92.8	15.67
8	20	4	6	11.5	1.01	28.2	147.2	12.11
9	20	5	3	30.2	1.15	53.2	137.6	7.25
10	22	5	3	15.7	0.26	16.8	94.4	21.87
11	22	6	3	12.4	0.67	33.2	98.2	15.43
DMSO[a]				100.0	0.83		100.0	10.47
AG[b]				7.0	0.11			
LET[c]							3.9	1.41

[a] Blank/negative control for both noncellular and cell-based bioassays. [b] Aminoglutethimide (AG), positive control for noncellular bioassay. [c] Letrozole (LET), positive control for cell-based bioassay.

Since most natural product drug discovery programs incorporate some form of secondary biological discrimination prior to intensive bioassay-guided fractionation, the unsaturated fatty acids were then subjected to IC_{50} (half-maximal inhibitory concentration) testing to determine if the fatty acids would act in a dose-dependent manner. The unsaturated fatty acids did, in fact, exhibit a typical dose-dependent response in the noncellular aromatase assay with values ranging from 16.8 μM for **10** to 53.2 μM for **9** (Table 1). On the basis of these results, even if extracts found to be active during screening are followed up by obtaining IC_{50} curves, the extracts may still be mistakenly considered for time-consuming activity-guided fractionation.

To determine if the unsaturated fatty acids inhibit aromatase in a more biologically relevant, cell-based assay, the unsaturated fatty acids were then tested at 100 μM in a secondary cell-based assay, using SK-BR-3 hormone-independent human breast cancer cells that overexpress the aromatase enzyme.[18] This assay measures the amount of tritiated water (^3H_2O) released as aromatase converts [1β-^{3}H]androst-4-ene-3,17-dione to estrone within cells. All unsaturated fatty acids were found to be inactive in this cell-based aromatase assay (Figure 1, Table 1). Interestingly, compounds **6** (147.6 PCA), **8** (147.2 PCA), and **9** (137.6 PCA) all had PCA values significantly higher than the negative control, dimethyl sulfoxide (DMSO). These high PCA values may indicate that these fatty acids are actually stimulating aromatase expression, possibly through their conversion to prostaglandin E_2 (PGE_2), which has been shown to upregulate aromatase expression and activity.[19] Aromatase is bound to the endoplasmic reticulum within cells throughout the female body, with the highest levels found in the ovaries, placenta of pregnant women, and adipose tissue.[16,17] The negative findings from this cell-based assay suggest that unsaturated fatty acids are not inhibiting aromatase and that the previously reported antitumorigenic effects of fatty acids on breast cancer cells are not the result of aromatase inhibition. Thus, natural product plant extracts containing fatty acids usually should not be considered for further aromatase bioassay-guided fractionation.

Accordingly, the findings of this study have shown that fatty acids can interfere with natural product screening results obtained in a noncellular microsomal radiometric aromatase assay. Fatty acids are a common component of human diets, and several fatty acids have been investigated for their potential clinical use as nutritional supplements and/or medicinal agents.[20] Epidemiological evidence has indicated that some fatty acids may be active against cancer, cardiovascular disease, autoimmune diseases, and various other conditions.[21,22] Numerous studies have examined the influence of various fatty acids on cellular and enzymatic processes in vitro, in vivo, and in clinical trials.[23,24] Although fatty acids may be biologically relevant and clinically useful, their almost ubiquitous occurrence in plant extracts and frequent positive response in noncellular assays necessitates the detection or removal of fatty acids during screening efforts and prior to large-scale bioassay-guided fractionation.

Various methods can be considered for dereplication of fatty acids in natural product extracts. Gas chromatography−mass spectrometry (GC-MS) and high-pressure liquid chromatography−mass spectrometry (HPLC-MS) are routinely used in the detection and/or separation of fatty acids for the purposes of microbial chemotaxonomy,[25] and both techniques can be readily employed for the detection of fatty acids in natural product extracts. GC-MS is the American Oil Chemists' Society standard method for

determination of fatty acid composition of marine oils[26] and can be adapted to detect fatty acids in active natural product extracts. HPLC-MS can be used to detect fatty acids in natural product extracts by comparison of retention times of known fatty acids with those of active extracts.[9] Removal of fatty acids from natural product extracts could be accomplished using a range of techniques. Defatting extracts with a suitable nonpolar solvent prior to bioassay screening should remove many fatty acids, but will leave the more polar fatty acids in the extract.[27] Centrifugal partition chromatography (CPC) has been proposed as a method for prefractionation of crude plant extracts, comparing the retention times of active fractions with the retention times of known fatty acids.[10] Aminopropyl-bonded silica solid phased extraction (SPE) has been adapted to separate fatty acids from natural product extracts, but also retains other carboxylic acids, some of which may be of interest to drug discovery research.[28] Capillary electrophoresis has also been employed to quickly separate fatty acids in human serum samples[29] and could be adapted for separation of fatty acids from natural product extracts. Alternatively, secondary evaluation of active extracts in compatible cell-based bioassays could be employed to validate potential hits identified in the enzyme-based bioassay prior to the expensive, time-consuming process of natural product bioassay-guided fractionation. The appropriate method of detection or removal of fatty acids will ultimately depend on available instrumentation and time constraints imposed by individual natural product drug discovery programs.

Fatty acids have been shown to interfere with many noncellular assays, providing positive results in initial screening, which either are refuted in secondary screening or are found to be the result of nonspecific, noncompetitive binding. In future natural product screening drug discovery projects, it is recommended that extracts active in noncellular bioassays should be evaluated for the presence of fatty acids prior to bioassay-guided fractionation.

Experimental Section

General Experimental Procedures. Pure fatty acids were purchased from Sigma-Aldrich (St. Louis, MO) comprising saturated fatty acids [myristic (**1**), pentadecanoic (**2**), palmitic (**3**), and stearic (**4**) acids] and unsaturated fatty acids [oleic (**5**), linoleic (**6**), α-linolenic (**7**), arachidonic (**8**), eicosapentaenoic (**9**), docosapentaenoic (**10**), and docosahexaenoic (**11**) acids]. Radiolabeled [1β-^{3}H]androst-4-ene-3,17-dione was purchased from NEN Life Science Products (Boston, MA). Radioactivity was counted on a LS6800 liquid scintillation counter (Beckman, Palo Alto, CA). Scintillation cocktail 3a70B was purchased from Research Prospect International Corporation (Mount Prospect, IL). SK-BR-3 hormone-independent human breast cancer cells were obtained from American Type Culture Collection (Rockville, MD). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Noncellular, Enzyme-Based Aromatase Bioassay. Human placental microsomes were obtained from human term placentas that were processed at 4 °C immediately after delivery from The Ohio State University Medical Center [OSU Institutional Review Board (IRB) protocol number 2002H0105, last approved in January 2005]. After washing the placenta with normal saline, connective and vascular tissues were removed. Microsomes were obtained from the remaining tissue as previously described.[30] Aliquots of microsomes were stored at −80 °C until required.

Fatty acids were originally screened at 20 μg/mL using a noncellular microsomal radiometric aromatase assay, performed as previously described.[15] Samples [fatty acids, DMSO as negative control, or 50 μM (±)-aminoglutethimide (AG) as positive control] were run in triplicate. Samples were added to 100 nM [1β-^{3}H]androst-4-ene-3,17-dione (400 000−450 000 dpm), 0.1 M potassium phosphate buffer (pH 7.0), 5% propylene glycol, and a NADPH regenerating system (containing 2.85 mM glucose-6-phosphate, 1.8 mM $NADP^+$, and 1.5 units of glucose-6-phosphate dehydrogenase). The reactions were initiated by adding 50 μg of microsomal aromatase, incubated in a shaking water bath at 37 °C, and quenched after 15 min using 2 mL of $CHCl_3$. Tubes were vortexed and then centrifuged for 5 min. The aqueous layer was removed from each tube and extracted two more times with $CHCl_3$ to afford an exhaustive extraction. An aliquot of the aqueous layer was then added to 3a70B scintillation cocktail for quantitation of the formation of ^3H_2O. Background values were determined using boiled, inactivated microsomal aromatase. Results are given as percent control activity (PCA) calculated using the formula

$$\text{PCA} = (\text{Sample dpm} - \text{DMSO dpm})/(\text{DMSO dpm} - \text{Boil dpm}) \times 100$$

where dpm is disintegrations per min and Boil is the background determined by inactivating the microsomal aromatase by boiling. IC_{50} values were determined for the unsaturated fatty acids by nonlinear regression using eight inhibitor concentrations ranging from 1 to 100 μM. IC_{50} dose−response curves were analyzed using Graphpad Prism (Version 3.0).

Cell-Based Aromatase Bioassay. Active fatty acids were further tested at 100 μM concentration in SK-BR-3 hormone-independent human breast cancer cells that overexpress aromatase, using a previously described methodology.[18,31] SK-BR-3 cell cultures were maintained in custom phenol red-free media containing MEM, Earle's salts, 1.5× amino acids, 2× nonessential amino acids, L-glutamine, and 1.5× vitamins (Life Technologies, Carlsbad, CA). The media was supplemented with 10% fetal bovine serum (heat inactivated for 30 min in a 56 °C water bath), 2 mM L-glutamine, and 20 mg/L gentamycin. Cells were grown to subconfluency in T-25 flasks under 5% carbon dioxide at 37 °C in a Hereaus CO_2 incubator. The medium was changed before treatment to contain DMEM/F12 media with 1.0 mg/mL human albumin (OSU Hospital Pharmacy, Columbus, OH), 5.0 mg/L human transferrin, and 5.0 mg/L bovine insulin.

Cells in T-25 flasks were treated with samples or 0.1% DMSO (negative control) or 10 nM letrozole (positive control) [in triplicate]. After 24 h, the medium was changed, 50 nM androstenedione with 2 μCi [1β-^{3}H]androst-4-ene-3,17-dione was added, and cells were incubated for 6 h. The reaction mixture was then removed followed by precipitation of proteins using 10% trichloroacetic acid at 42 °C for 20 min. The mixture was briefly centrifuged and the aqueous layer was extracted three times with $CHCl_3$ to remove unused substrate. The aqueous layer was subsequently treated with 1% dextran-coated charcoal. An aliquot of the aqueous layer was added to 3a70B scintillation cocktail for quantitation of the formation of ^3H_2O. Results were corrected for blanks and for the amount of cells in each flask, determined by trypsinizing cells and analyzed using the diphenylamine DNA assay adapted to a 96-well plate format.[18,31] Results were expressed as picomoles of ^3H_2O formed per hour of incubation per million live cells (pmol/h/10^6 cells).

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Supporting Information Available: IC_{50} curves from the noncellular, enzyme-based aromatase assay for the unsaturated fatty acids found to be active during screening, including oleic, linoleic, α-linolenic, arachidonic, eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids, are available free of charge via the Internet at http://pubs.acs.org.

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