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Inhibition of catechol-*O*-methyltransferase increases estrogen-DNA adduct formation

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Abstract

The association found between breast cancer development and prolonged exposure to estrogens suggests that this hormone is of etiologic importance in the causation of the disease. Studies on estrogen metabolism, formation of DNA adducts, carcinogenicity, cell transformation and mutagenicity have led to the hypothesis that reaction of certain estrogen metabolites, predominantly catechol estrogen-3,4-quinones, with DNA forms depurinating adducts [4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua]. These adducts cause mutations leading to the initiation of breast cancer. Catechol-*O*-methyltransferase (COMT) is considered an important enzyme that protects cells from the genotoxicity and cytotoxicity of catechol estrogens, by preventing their conversion to quinones. The goal of the present study was to investigate the effect of COMT inhibition on the formation of depurinating estrogen-DNA adducts. Immortalized human breast epithelial MCF-10F cells were treated with 4-OHE_2 (0.2 or 0.5 μM) for 24 h at 120, 168, 216, and 264 h post-plating or one time at 1–30 μM 4-OHE_2 with or without the presence of COMT inhibitor (Ro41-0960). The culture media were collected at each point, extracted by solid-phase extraction and analyzed by HPLC connected with a multichannel electrochemical detector. The results demonstrate that MCF-10F cells oxidize 4-OHE_2 to $E_1(E_2)$-3,4-Q, which react with DNA to form the depurinating N3Ade and N7Gua adducts. The COMT inhibitor Ro41-0960 blocked the methoxylation of catechol estrogens, with concomitant 3–4 fold increases in the levels of the depurinating adducts. Thus, low activity of COMT leads to higher levels of depurinating estrogen-DNA adducts that can induce mutations and initiate cancer.

Keywords

estrogen metabolism; estrogen protective enzymes; COMT inhibition; depurinating estrogen-DNA adducts

Introduction

Prolonged exposure of women to high estrogen levels is associated with an elevated incidence of breast cancer [1–5]. Experiments on estrogen metabolism [6–10], formation of DNA adducts [11–17], mutagenicity [17–21], cell transformation [22–24] and carcinogenicity [25–28] have led to the hypothesis that certain estrogen metabolites, predominantly catechol estrogen-3,4-quinones, react with DNA to cause the mutations leading to the initiation of cancer (Fig. 1)

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[17]. The reaction of estrone(estradiol)-3,4-quinones [$E_1(E_2)$-3,4-Q], derived from 4-$OHE_1(E_2)$, with DNA produces predominantly depurinating adducts and very small amounts of stable adducts [11,13,14,29].

In extrahepatic tissues, cytochrome P450 (CYP)1A1 and CYP1B1 predominantly metabolize the natural estrogens E_1 and E_2 to 2- and 4- catechol estrogens (CE), respectively [30–32], which can be competitively oxidized to their respective semiquinones and quinones. In general, the CE are inactivated by conjugating reactions, such as glucuronidation and sulfation. A common pathway of inactivation in extrahepatic tissues, however, occurs by *O*-methylation catalyzed by the ubiquitous catechol-*O*-methyltransferase (COMT) [33]. If the formation of E_1 and E_2 is excessive, due to overexpression of aromatase and/or the presence of excess sulfatase that converts stored E_1 sulfate to E_1, increased formation of CE is expected (Fig. 1). In particular, the presence and/or induction of CYP1B1 and other 4-hydroxylases could dramatically increase the formation of 4-$OHE_1(E_2)$. Thus, conjugation of 4-$OHE_1(E_2)$ via methylation can become insufficient, and competitive oxidation of 4-$OHE_1(E_2)$ to $E_1(E_2)$-3,4-Q could be more abundant [34]. The increased level of quinones would generate more reaction with DNA at the N-3 of adenine (Ade) and N-7 of guanine (Gua) to form depurinating adducts (Fig. 1) [17,29,34]. These adducts are lost from DNA by destabilization of the glycosyl bond. The apurinic sites generated in the DNA can produce mutations by error-prone repair [18–20], which in turn can lead to initiation of cancer.

The Phase II enzyme COMT is considered to be a key enzyme in decreasing the effects of 4-$OHE_1(E_2)$ by converting the catechol estrogens into the corresponding methoxy derivatives [33]. COMT is an intracellular enzyme that is present as both soluble and membrane-bound forms encoded by the same gene with different transcription start sites [35]; the soluble form is the major one in most organs [33]. COMT activity can be altered by either endogenous factors, such as genetic polymorphisms and levels of expression, or exogenous factors such as inhibition by environmental compounds. Genetic epidemiology studies have proposed a possible correlation between the low activity allele ($COMT^{LL}$) and increased breast cancer risk [36–38].

COMT activity can be inhibited by many natural and synthetic compounds [33,39,40]. Ro41-0960 is a nitrocatechol-type inhibitor of COMT that inhibits methylation of catechol estrogens. It is a poor substrate for COMT, but binds tightly to catalytic sites of the enzyme, thus inhibiting methylation of other substrates without depleting cofactors [41,42]. We hypothesize that COMT inhibition decreases inactivation of CE, which may in turn lead to increased formation of CE-Q and DNA damage that initiates cancer.

To fully understand how estrogens can become carcinogenic in the human breast through metabolic activation to CE-Q an experimental system is required in which estrogens or their metabolites (e.g., 4-OHE_2) would induce transformation phenotypes in human breast epithelial cells *in vitro* that are indicative of neoplasia. Data from a recent study showed that successive treatment of MCF-10F cells with 4-OHE_2 induced mutations, cell transformation and cancer [21,24]. The ERα-negative MCF-10F cell line is a good experimental model for researching the carcinogenicity and mutagenic potential of 4-OHE_2. To investigate the implications of possible COMT inhibition by Ro41-0960 and increased formation of depurinating adducts, the cells were preincubated with 3 μM Ro41-0960 and then treated with 4-OHE_2 (0.2–30 μM) for 24 h. The profile of 4-OHE_2 metabolites, conjugates and depurinating DNA adducts was determined in cell culture medium by HPLC equipped with a multichannel electrochemical detector (ECD) and validated by ultraperformance liquid chromatography (UPLC)-MS/MS techniques. This is the first report on the metabolic profile of 4-OHE_2 in MCF-10F cells treated in a dose-response manner

Materials and Methods

Chemicals and Reagents

4-OHE_2 and all standards were synthesized in our laboratory, as previously described [13, 43–45]. Ro41-0960 and all other chemicals were purchased from Sigma (St. Louis, MO). MCF-10F cells were obtained from the ATCC (Rockville, MD).

Cell culture and treatment

MCF-10F cells were cultured in phenol red DMEM/F12 (1:1) medium containing 20 ng/ml epidermal growth factor, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, 5 % horse serum and 100 μg/ml penicillin/streptomycin mixture and maintained in a humidified incubator at 37 ºC and 5% CO_2. Estrogen-free medium was prepared in phenol red-free DMEM/F12 medium with charcoal-stripped fetal bovine serum (FBS). To keep the concentration of DMSO the same (0.001%) in all experiments, different stock solutions of 4-OHE_2 (0.2–30 mM) were prepared. A stock of 9 mM Ro41-0960 was prepared in ethanol.

The MCF-10F cells (2.5×10^5 cells) were seeded for 48 h in estrogen-containing medium. The medium was changed to estrogen-free medium and the cells were grown for another 72 h. To investigate the direct relationship of COMT inhibition on the formation of depurinating adducts, the cells were first treated with 3 μM Ro41-0960 for 1 h and then treated once with various concentrations of 4-OHE_2 (0–30 μM) for 24 h.

For multiple treatment experiments, 1.0×10^5 MCF-10F cells were seeded and treated with 0.2 or 0.5 μM 4-OHE_2 for 24 h at 120, 168, 216 and 264 h post-seeding. Cell cultures were or were not pre-incubated with Ro41-0960 for 1 h prior to the addition of 4-OHE_2. After each treatment, the medium was removed, ascorbic acid was added (2 mM final concentration) to prevent further oxidation of desired compounds, and the mixture was processed immediately. Media from four T-150 flasks of MCF-10F cells treated with 10 μl DMSO and 3.3 μl of ethanol were used as controls.

Sample preparation and analysis by HPLC-ECD and by UPLC-MS/MS

i. Sample Preparation—Culture media from four flasks (40 mL) were processed by passing through Varian C8 Certify II cartridges (Varian, Harbor City, CA). The cartridges were pre-equilibrated by sequentially passing 1 ml of methanol, distilled water, and potassium phosphate buffer (100 mM, pH 8) through them. Culture media were adjusted with 1 ml of 1 M potassium phosphate buffer to pH 8.0 and passed through the cartridges. After washing with 200 μl of the phosphate buffer, the analytes were eluted with 1 ml of elution buffer [methanol:acetonitrile:water: trifluoroacetic acid (8:1:1:0.1)] and evaporated by using a Jouan concentrator (Thermo Scientific, Waltham, MA). The residue was resuspended in 150 μl of methanol/water (1:1) and filtered through a 5000-MW cut-off filter (Millipore, Bedford, MA).

ii. HPLC—Analyses of all samples were conducted on an HPLC system equipped with dual ESA Model 580 solvent delivery modules, an ESA Model 540 auto-sampler and a 12-channel CoulArray electrochemical detector (ESA, Chelmsford, MA). The two mobile phases used were A: acetonitrile:methanol:buffer:water (15:5:10:70) and B: acetonitrile:methanol: buffer:water (50:20:10:20). The buffer was a mixture of 0.25 M citric acid and 0.5 M ammonium acetate in triple-distilled water, and the pH was adjusted to 3.6 with acetic acid. The 95-μl injections were carried out on a Phenomenex Luna-2 C-18 column (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA), initially eluted isocratically at 90% A/10% B for 15 min, followed by a linear gradient to 90% B in the next 40 min, and held there for 5 min (total 50 min gradient) at a flow rate of 1 ml/min and a temperature of 30 °C. The serial array of 12 coulometric electrodes was set at potentials of -35, 10, 70, 140, 210, 280, 350, 420, 490, 550,

620 and 690 mV. The system was controlled and the data were acquired and processed using the CoulArray software package (ESA). Peaks were identified by both retention time and peak height ratios between the dominant peak and the peaks in the two adjacent channels. The metabolites, conjugates and depurinating adducts were quantified by comparison of peak response ratios with known amounts of standards.

iii. UPLC-MS/MS—Some of the results from HPLC-ECD determinations were validated by analyzing the same samples on a Waters Acquity UPLC equipped with a MicroMass QuattroMicro triple stage quadrupole mass spectrometer (Waters, Milford, MA). The 10-μl injections were carried out on a Waters Acquity UPLC $BEHC_{18}$ column (1.7 μm, 1 × 100 mm). The instrument was operated in the positive electrospray ionization mode. All aspects of system operation, data acquisition and processing were controlled using QuanLynx v4.0 software (Waters). The column was eluted starting with 5% acetonitrile in water (0.1% formic acid) for 1 min at a flow rate of 150 μl/min; then a gradient to 55% acetonitrile in 10 min was run. Ionization was achieved using the following settings: capillary voltage 3 kV; cone voltage 15–40 V; source block temperature 100 °C; desolvation temperature 200 °C, with a nitrogen flow of 400 l/h. Nitrogen was used as both the desolvation and auxiliary gas. Argon was used as the collision gas. Three-point calibration curves were run for each standard. Triplicate samples were analyzed for each data point.

Results and Discussion

To examine the profile of estrogen metabolism in MCF-10F cells, an HPLC method with a CoulArray ECD [10] was used to quantify the relative concentrations of estrogen metabolites, conjugates and depurinating DNA adducts. Standard solutions of each compound were combined to generate equimolar mixtures containing varying concentrations of each estrogen standard and injected onto the column. These standard solutions were then used to generate calibration curves. Standard curves were linear between 125 and 500 pg/μl. The limit of detection under the conditions of analysis was ~10 pg/μl on column (for 95-μl injection volume).

To determine the effect of COMT inhibition on 4-OHE_2 metabolism, MCF-10F cells were pretreated with 3 μM Ro41-0960 for 1 h and then exposed to different concentrations of 4-OHE_2. This concentration of the inhibitor reduced COMT activity by 90–95% at 24 h, as assessed by methylation of 4-OHE_2 (data not shown).

To mimic intermittent exposure of immortalized MCF-10F cells to estrogen [22,23], the cells were successively treated with 0.2 or 0.5 μM 4-OHE_2 twice a week for two weeks (Tables 1 and 2). The cell culture medium was removed 24 h after each treatment and processed for analysis of estrogen metabolites, conjugates and depurinating DNA adducts. Treatment with the COMT inhibitor decreased the concentration of methoxylated conjugates formed from 0.2 μM 4-OHE_2 to undetectable levels and increased the recovery of 4-$OHE_1(E_2)$ 6–12-fold (Table 1).

In the absence of the inhibitor, 4-$OCH_3E_1(E_2)$ was the major product of metabolism from 0.2 μM 4-OHE_2. Small, but increasing amounts of the GSH and Cys conjugates were detected after successive treatments with 4-OHE_2. The 4-$OHE_1(E_2)$-2-Cys conjugates are presumably obtained by mercapturic acid biosynthesis from the 4-$OHE_1(E_2)$-2-SG [46] and by conjugation of $E_1(E_2)$-3,4-Q with the 0.4 mM Cys present in the culture medium. The presence of Cys in the medium diminishes the significance of these results. The depurinating adducts were also detected in small amounts after the 3rd and 4th treatments (Table 1). In the presence of the COMT inhibitor, the methoxy conjugates were undetectable, whereas the GSH and Cys conjugates, as well as the two depurinating DNA adducts were increased (Table 1). In the

presence of the COMT inhibitor, low levels of both adducts were detected after the 2nd treatment. After the 3rd treatment, the adducts could be quantified if the inhibitor was present. After the 4th treatment, the presence of the inhibitor resulted in approximately twice as much N3Ade, although the level of the N7Gua adduct was about the same as without the inhibitor.

When the cells were treated with 0.5 μM 4-OHE_2, inclusion of the COMT inhibitor increased the level of 4-$OHE_1(E_2)$ in the medium 5–8 fold and the 4-$OCH_3E_1(E_2)$ was undetectable. The levels of the GSH and Cys conjugates increased a little in the presence of the COMT inhibitor, especially at the early time points. With the inhibitor, the level of N3Ade adduct was increased after the 2nd, 3rd and 4th treatments with 4-OHE_2, and the N7Gua adduct was quantifiable after the 3rd and 4th treatments. Statistical comparison of adduct levels could be carried out only for N3Ade after the 4th treatment; the presence of the COMT inhibitor led to a significant increase ($p < 0.01$).

When MCF-10F cells were incubated with 1–30 μM 4-OHE_2 for 24 h, a dose-response was observed (Table 3, Fig. 2). Increased concentrations of 4-OHE_2 produced a large increase in 4-$OCH_3E_1(E_2)$ formed. When the COMT inhibitor was present, however, formation of 4-$OCH_3E_1(E_2)$ decreased 98–99% ($p < 0.003$). In parallel, the higher concentrations of 4-OHE_2 yielded an increase in the depurinating N3Ade and N7Gua adducts (Table 3, Fig. 2). A more dramatic 3 to 4-fold increase occurred when Ro41-0960 was present. In the presence of the inhibitor the levels of both adducts were significantly different, $p < 0.05$. The level of 4-$OHE_1(E_2)$-2-SG conjugate was low and was unchanged by the presence of the inhibitor. The Cys conjugate, in contrast, had a dose-response based on the concentration of 4-OHE_2 and was 4-fold higher in the presence of the COMT inhibitor (Table 3, Fig. 2). In the presence of the COMT inhibitor, we speculate that the overall recovery of estrogen compounds is much lower because the catechol estrogens are oxidized to $E_1(E_2)$-3,4-Q, which readily react with the SH groups of Cys and amino group of lysine in proteins.

As shown in Figure 1, a balanced estrogen metabolism involves conversion of 4-OHE_2 to its methoxy derivative. This process is catalyzed by the enzyme COMT. A competing pathway for 4-OHE_2 is its oxidation to E_2-3,4-semiquinone and then to E_2-3,4-Q. Two other pathways can inhibit the formation of depurinating DNA adducts formed by reaction of $E_1(E_2)$-3,4-Q with DNA. One is the reduction of the quinone to the CE catalyzed by quinone reductase (Fig. 1) [47,48]. The second pathway is the reaction of the quinone with GSH.

In the present study, we have found that in MCF-10F cells increasing concentrations of 4-OHE_2 afford higher levels of the depurinating adducts (Fig. 2, Table 3). At the same time, very large amounts of 4-$OCH_3E_1(E_2)$ are observed, indicating that large amounts of COMT are present in the cells. When the COMT inhibitor was present, inhibition of CE methylation was almost total and the levels of the N3Ade and N7Gua adducts increased four-fold (Fig. 2, Table 3). From these studies inhibition of COMT activity clearly unbalances estrogen metabolism toward excessive formation of $E_1(E_2)$-3,4-Q. As part of this imbalance, greater formation of hydroxyl radicals occurs. This is demonstrated by formation of 8-hydroxy-2′-deoxyguanosine, which is derived from the increased redox cycling between the estrogen semiquinones and quinones (Fig. 1) [49].

Another important factor is excessive formation of 4-$OHE_1(E_2)$ as a major metabolite of $E_1(E_2)$, catalyzed by CYP1B1 [30–32]. Minimization of estrogen-DNA adduct formation occurs when COMT is present at high levels because methoxylation of 4-$OHE_1(E_2)$ is one of the key elements in reducing adduct formation. This important role of COMT in protecting cells from cancer initiation by estrogens suggests that COMT polymorphisms could have a significant effect on cancer incidence. For example, the common val108met polymorphism decreases COMT activity 3–4-fold [50,51]. Thus, persons homozygous for this polymorphism

could be at increased risk for estrogen-induced cancers. The critical events described above are extremely useful in determining the agents that can minimize the formation of estrogen-DNA adducts, thereby inhibiting the initiation of breast and other human cancers.

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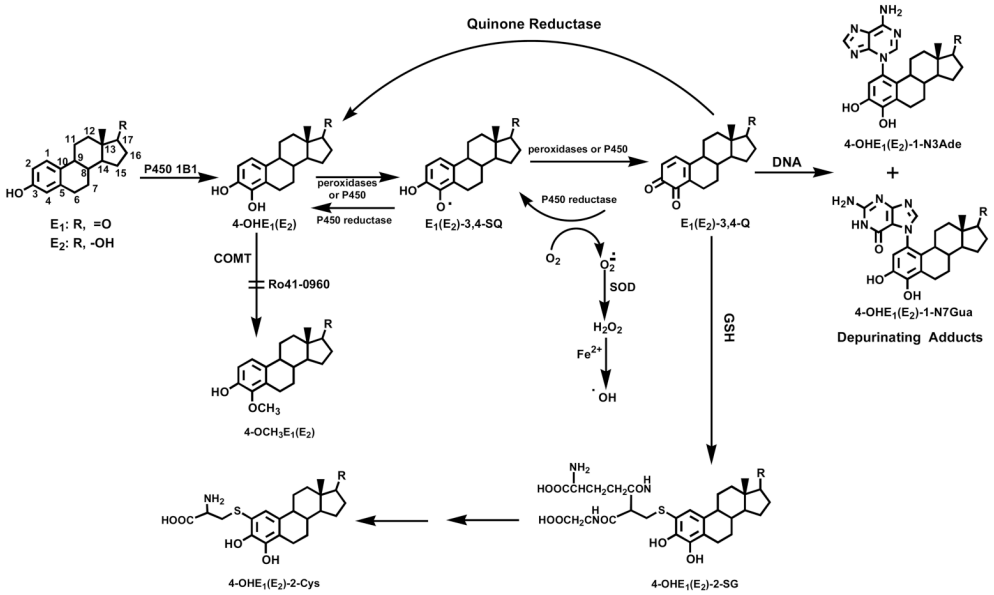

Fig. 1.
Major pathway of estrogen activation in the formation of depurinating estrogen-DNA adducts.

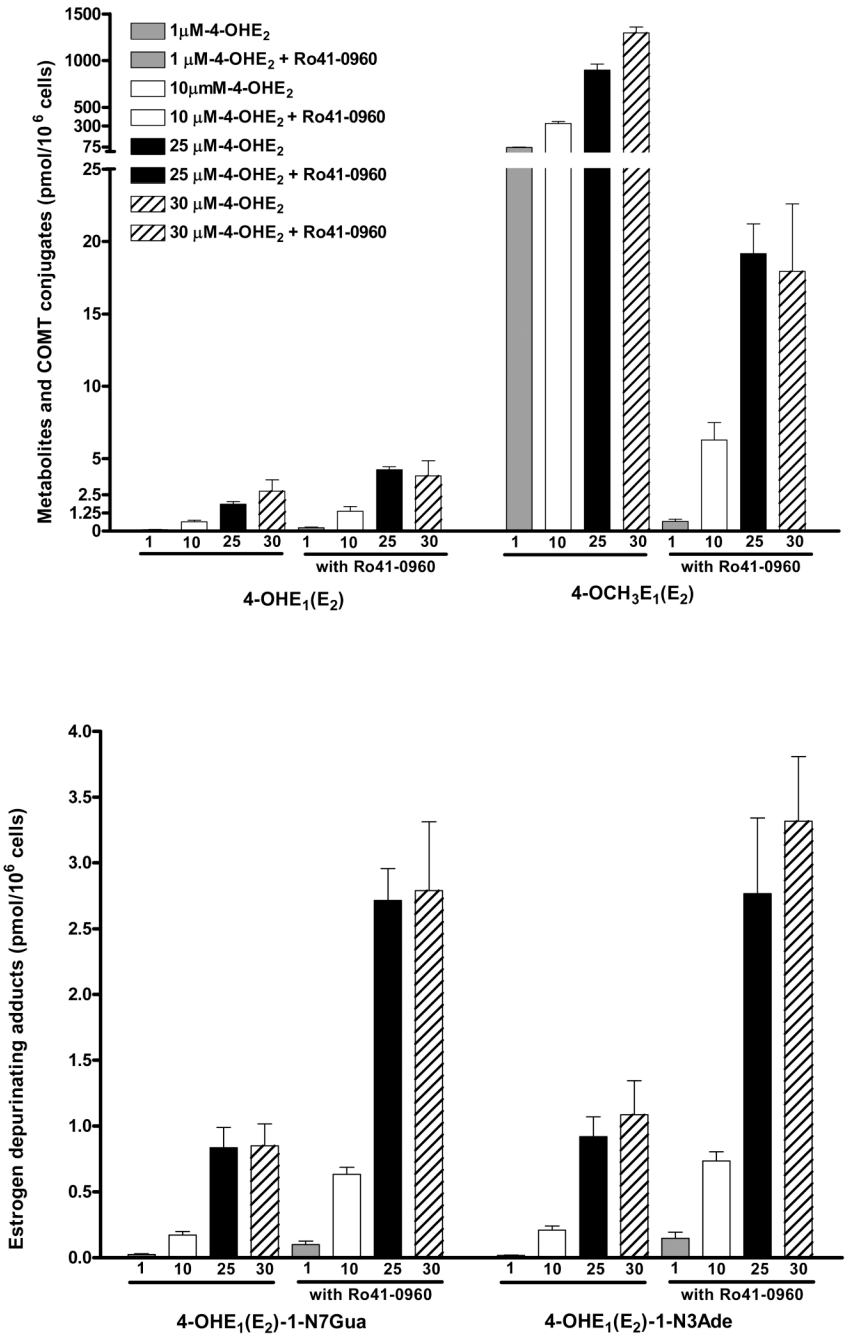

Fig. 2.

Incubation of MCF-10F cells with 1–30 μM 4-OHE_2 with or without 3 μM Ro41-0960 (COMT inhibitor) for 24 h.

Table 1

Intermittent incubation of MCF-10F cells with 0.2 μM 4-OHE_2 with or without 3 μM Ro41-0960[1]

Detected Compounds[2]	4-OHE_2 treatment				4-OHE_2+ Ro41-0960 treatment			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
	pmol/10^6cells							
4-$OHE_1(E_2)$	0.08 ± 0.02	0.22 ± 0.02	0.27 ± 0.08	0.31 ± 0.04	0.98 ± 0.01	1.64 ± 0.03	1.81 ± 0.11	1.91 ± 0.20
4-$OCH_3E_1(E_2)$	0.10 ± 0.01	0.11 ± 0.04	0.39 ± 0.21	0.76 ± 0.12	nd[3]	nd	nd	nd
4-OH $E_1(E_2)$-2-SG	nd	nd	nd	0.01 ± 0.01	nd	nd	0.09 ± 0.03	0.36 ± 0.09
4-OH $E_1(E_2)$-2-Cys	nd	nd	nd	0.18 ± 0.03	0.12 ± 0.03	0.16 ± 0.03	1.66 ± 0.35	2.35 ± 0.96
4-OH $E_1(E_2)$-1-N3Ade	nd	nd	loq[4]	0.30 ± 0.07	nd	loq	0.55 ± 0.13	0.59 ± 0.07
4-OH $E_1(E_2)$-1-N7Gua	nd	nd	loq	0.81 ± 0.12	nd	loq	0.44 ± 0.11	0.85 ± 0.12

[1] 4-OHE_2 was incubated with MCF-10F cells at 37 °C for 24 h in the presence or absence of Ro41-0960 (COMT inhibitor) twice weekly for two weeks.

[2] The compounds were identified and quantified by HPLC-ECD, and values are an average of three replicates.

[3] Not detected.

[4] Although the compound was identified, it could not be quantified due to the limit of quantification.

Table 2

Intermittent incubation of MCF-10F cells with 0.5 μM 4-OHE_2 with or without 3 μM Ro41-0960[1]

Detected Compounds[2]	4-OHE_2 treatment				4-OHE_2 + Ro41-0960 treatment			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
	pmol/10^6cells							
4-$OHE_1(E_2)$	0.14 ± 0.02	0.46 ± 0.02	0.49 ± 0.04	0.47 ± 0.04	1.14 ± 0.01	2.30 ± 0.03	2.49 ± 0.98	2.58 ± 0.96
4-OCH_3 $E_1(E_2)$	0.25 ± 0.01	2.58 ± 0.54	7.64 ± 0.40	5.18 ± 0.11	nd[3]	nd	nd	nd
4-OH $E_1(E_2)$-2-SG	nd	nd	0.27 ± 0.01	0.62 ± 0.04	0.04 ± 0.03	0.34 ± 0.03	0.38 ± 0.02	0.60 ± 0.06
4-OH $E_1(E_2)$-2-Cys	nd	0.32 ± 0.03	0.75 ± 0.42	1.38 ± 0.04	0.75 ± 0.01	1.18 ± 0.03	1.16 ± 0.05	1.49 ± 0.01
4-OH $E_1(E_2)$-1-N3Ade	nd	nd	loq[4]	0.25 ± 0.12	nd	0.21 ± 0.01	0.55 ± 0.01	0.83 ± 0.09
4-OH $E_1(E_2)$-1-N7Gua	nd	nd	loq	loq	nd	loq	0.81 ± 0.12	0.70 ± 0.19

[1] 4-OHE_2 was incubated with MCF-10F cells at 37 °C for 24 h in the presence or absence of Ro41-0960 (COMT inhibitor) twice weekly for two weeks.

[2] The compounds were identified and quantified by HPLC-ECD, and values are an average of three replicates.

[3] Not detected.

[4] Although the compound was identified, it could not be quantified due to the limit of quantification.

Table 3

Incubation of MCF-10F cells with 1–30 μM of 4-OHE_2 with or without 3 μM Ro41-0960 for 24 hrs at 37 °C

Compounds Detected	4-OHE_2 treatment				4-OHE_2 + Ro41-0960 treatment			
	1 μM	10 μM	25 μM	30 μM	1 μM	10 μM	25 μM	30 μM
	pmol/10^6 cells							
4-$OHE_1(E_2)$	0.10 ± 0.03	0.63 ± 0.21	1.86 ± 0.3	2.75 ± 1.36	0.60 ± 0.05	3.64 ± 0.57	4.91 ± 0.85	5.81 ± 1.02
4-$OCH_3E_1(E_2)$	70 ± 5	340 ± 34	899 ± 112	1297 ± 110	0.66 ± 0.26	6.37 ± 2.17	19.2 ± 3.5	17.9 ± 2.3
4-$OHE_1(E_2)$-2-SG	0.08 ± 0.03	0.35 ± 0.04	0.54 ± 0.23	1.1 ± 0.36	0.14 ± 0.02	0.47 ± 0.13	0.59 ± 0.18	0.87 ± 0.32
4-$OHE_1(E_2)$-2-Cys	0.25 ± 0.03	0.62 ± 0.12	3.89 ± 0.49	7.9 ± 1.93	0.3 ± 0.21	1.51 ± 0.56	16.1 ± 4.7	33.3 ± 6.4
4-$OHE_1(E_2)$-1-N7Gua	0.02 ± 0.01	0.17 ± 0.05	0.84 ± 0.27	0.85 ± 0.29	0.10 ± 0.05	0.63 ± 0.09	2.71 ± 0.42	2.79 ± 0.90
4-$OHE_1(E_2)$-1-N3Ade	0.02 ± 0.01	0.21 ± 0.05	0.92 ± 0.26	1.09 ± 0.45	0.15 ± 0.08	0.74 ± 0.12	2.77 ± 1.00	3.32 ± 0.85