

Catechol-*O*-Methyltransferase (COMT)-mediated Metabolism of Catechol Estrogens: Comparison of Wild-Type and Variant COMT Isoforms¹

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ABSTRACT

The oxidative metabolism of 17 β -estradiol (E2) and estrone (E1) to catechol estrogens (2-OHE2, 4-OHE2, 2-OHE1, and 4-OHE1) and estrogen quinones has been postulated to be a factor in mammary carcinogenesis. Catechol-*O*-methyltransferase (COMT) catalyzes the methylation of catechol estrogens to methoxy estrogens, which simultaneously lowers the potential for DNA damage and increases the concentration of 2-methoxyestradiol (2-MeOE2), an antiproliferative metabolite. We expressed two recombinant forms of COMT, the wild-type (108Val) and a common variant (108Met), to determine whether their catalytic efficiencies differ with respect to catechol estrogen inactivation. The His-tagged proteins were purified by nickel-nitrilo-triacetic acid chromatography and analyzed by electrophoresis and Western immunoblot. COMT activity was assessed by determining the methylation of 2-OHE2, 4-OHE2, 2-OHE1, and 4-OHE1, using gas chromatography/mass spectrometry for quantitation of the respective methoxy products. In the case of 2-OHE2 and 2-OHE1, methylation occurred at 2-OH and 3-OH groups, resulting in the formation of 2-MeOE2 and 2-OH-3-MeOE2, and 2-MeOE1 and 2-OH-3-MeOE1, respectively. In contrast, in the case of 4-OHE2 and 4-OHE1, methylation occurred only at the 4-OH group, yielding 4-MeOE2 and 4-MeOE1, respectively. Individual and competition experiments revealed the following order of product formation: 4-MeOE2 > 4-MeOE1 \gg 2-MeOE2 > 2-MeOE1 > 2-OH-3-MeOE1 > 2-OH-3-MeOE2. The variant isoform differed from wild-type COMT by being thermolabile, leading to 2–3-fold lower levels of product formation. MCF-7 breast cancer cells with the variant COMT 108Met/Met genotype also displayed 2–3-fold lower catalytic activity than ZR-75 breast cancer cells with the wild-type COMT 108Val/Val genotype. Thus, inherited alterations in COMT catalytic activity are associated with significant differences in catechol estrogen and methoxy estrogen levels and, thereby, may contribute to interindividual differences in breast cancer risk associated with estrogen-mediated carcinogenicity.

INTRODUCTION

COMT³ is an enzyme that catalyzes the transfer of a methyl group from the methyl donor SAM to one hydroxyl moiety of the catechol ring of a substrate (1). Physiological substrates of COMT include catecholamine neurotransmitters and the catechol estrogens, produced by cytochrome P-450-mediated metabolism of E2 and E1 (2, 3). Cell fractionation and immunological studies have shown that the enzyme occurs in two distinct forms, in the cytoplasm as a soluble protein (S-COMT) and in association with membranes as a membrane-bound

form (MB-COMT; Refs. 4–6). The amino acid sequence of S-COMT and MB-COMT is identical, except for an NH₂-terminal extension of 50 hydrophobic amino acids in MB-COMT that serves as an anchor to the membrane (7, 8). Genetic studies have demonstrated that S-COMT and MB-COMT are encoded by a single gene at 22q11.2 containing six exons, of which exons 1 and 2 are noncoding. A proximal promoter gives rise to the 1.3-kb S-COMT mRNA, whereas a distal promoter gives rise to the 1.5-kb MB-COMT mRNA (9). The MB-COMT and S-COMT proteins occur constitutively but differ in tissue expression. S-COMT is the predominant form in virtually all tissues, whereas MB-COMT generally accounts for ~10% of total enzyme activity (4, 10). For example, S-COMT constituted >90% of total COMT activity in normal breast tissue as well as MCF-7 breast cancer cells (9).

Lachman *et al.* (11) identified a G→A polymorphism in the *COMT* gene that is associated with a valine→methionine substitution in both the MB-form (codon 158) and S-form (codon 108). Using 3,4-dihydroxybenzoic acid as substrate, Syvanen *et al.* (12) determined that COMT activity in red blood cells from individuals with the homozygous *Met/Met* genotype was ~60% lower than that of homozygous *Val/Val* individuals, *i.e.*, 0.21–0.43 versus 0.55–1.03 pmol · min⁻¹ · mg⁻¹ protein. Heterozygotes showed intermediate activity. Although it is likely that the Val→Met polymorphism also affects catechol estrogen metabolism, it is not known whether the effect is similar for each of the four main catechol estrogens, *i.e.*, 2-OHE2, 2-OHE1, 4-OHE2, and 4-OHE1. It will be important to answer this question because experimental and epidemiological evidence suggests that the COMT-mediated reaction holds significant implications for estrogen-induced carcinogenesis and breast cancer development:

(a) Two of three molecular epidemiological studies indicate that the *Val/Val*, *Val/Met*, and *Met/Met* genotypes may be associated with differences in risk of developing breast cancer (13–15). Another study linked *COMT* genotypes to progression of breast cancer to the metastatic state (16).

(b) By inactivating catechol estrogens, COMT reduces the level of 2-OH and 4-OH estrogen metabolites, thereby lowering the potential for mutagenic damage through DNA adduct formation or through superoxide and hydroxy radicals arising from catechol estrogen quinone-semiquinone redox cycling (17, 18).

(c) 2-MeOE2, which is produced by COMT from 2-OHE2, has been shown to inhibit the proliferation of breast cancer cells *in vitro* and *in vivo* (19, 20). The inhibitory effect of 2-MeOE2 appears to be attributable to several mechanisms including the disruption of microtubule function, induction of apoptosis, and inhibition of angiogenesis (21–24). Oral administration of 2-MeOE2 (75 mg/kg) for 1 month suppressed the growth of human breast cancer in mice by 60% without toxicity (25). Thus, 2-MeOE2 (but not 4-MeOE2) appears to be an endogenous estrogen metabolite that inhibits mammary carcinogenesis (26).

In light of these considerations, we have cloned and expressed the wild-type and variant forms of COMT to determine whether the catalytic efficiency of the Met variant differs from that of the *Val* wild type with regard to the inactivation of E2 versus E1 catechol estrogens, the inactivation of 2-OH versus 4-OH metabolites, singly and in combination, and the rate of production of 2-MeOE2 from 2-OHE2.

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³ The abbreviations used are: COMT, catechol-*O*-methyltransferase; S-COMT, soluble form of COMT; MB-COMT, membrane-bound form of COMT; SAM, S-adenosyl-L-methionine; H:C, heated:control; CYP1A1, cytochrome P450 1A1; CYP1B1, cytochrome P450 1B1; E2, 17 β -estradiol; 2-OHE2, 2-hydroxyestradiol; 4-OHE2, 4-hydroxyestradiol; 2-MeOE2, 2-methoxyestradiol; 2-OH-3-MeOE2, 2-hydroxy-3-methoxyestradiol; 4-MeOE2, 4-methoxyestradiol; E1, estrone; 2-OHE1, 2-hydroxyestrone; 4-OHE1, 4-hydroxyestrone; 2-MeOE1, 2-methoxyestrone; 2-OH-3-MeOE1, 2-hydroxy-3-methoxyestrone; 4-MeOE1, 4-methoxyestrone; ScFv, single chain fragment variable; ICELISA, immune complex enzyme-linked immunosorbent assay; GST, glutathione S-transferase; NFD, nonfat dry milk; HRP, horseradish peroxidase.

We have also compared the activities of wild-type and variant COMT in breast cancer cell lines ZR-75 and MCF-7, respectively.

MATERIALS AND METHODS

Chemicals. Catechol estrogens (2-OHE2, 2-OHE1, 4-OHE2, and 4-OHE1) and methoxyestrogens (2-MeOE2, 2-MeOE1, 4-MeOE2, 4-MeOE1, 2-OH-3-MeOE2, 2-OH-3-MeOE1, 2-MeO-3-MeOE2, and 2-MeO-3-MeOE1) were obtained from Steraloids (Newport, RI). Deuterated E2 (E2-2, 4, 16, and 16-d4) was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada).

Cell Lines. Breast cancer cell lines ZR-75 and MCF-7 were obtained from the American Type Culture Collection (Rockville, MD) and grown under recommended culture conditions. DNA was isolated using a DNA extraction kit (Genra, Minneapolis, MN).

DNA Polymorphism Analysis. COMT was amplified with primers C1 (5'-GCCGCCATACCCAGCGGATGGTGGATTTCGCTGTC) and C2 (5'-GTTTTCAGTGAACGTGGTGTG). PCR was carried out in a total volume of 100 μ l containing 0.5 μ g of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each of the four deoxyribonucleotides, AmpliTaq DNA polymerase (2.5 units; Roche Diagnostics, Indianapolis, IN), and each primer at 25 μ M. Amplification conditions consisted of an initial denaturing step followed by 30 cycles of 95°C for 30 s, 64°C for 1 min, and 72°C for 6 min. A sample of the 160-bp PCR product was size fractionated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. A portion (10 μ l) of the PCR product was subjected to restriction digest with *Bsp*HI (New England Biolabs, Beverly, MA) at 37°C for 1 h. The digestion products were electrophoresed in a 4% low melting agarose gel (Amresco, Solon, OH) and visualized by ethidium bromide staining.

Expression and Purification of Recombinant S-COMT. Breast cancer cell lines ZR-75 (*Val/Val*) and MCF-7 (*Met/Met*) served as source for wild-type and variant S-COMT cDNA, respectively. Primers were designed to contain *Sac*I and *Sal*I sites, respectively, at their 5' ends to allow amplification of wild-type and variant S-COMT cDNA and ligation of the PCR product into vector pQE-30 (QIAGEN, Valencia, CA), which encodes an NH₂-terminal hexahistidine tag for subsequent purification (27). Each ligated vector/insert was transformed into XL1-Blue cells for amplification. The amplified plasmid DNA was then transformed into *Escherichia coli* strain DH5 α F'Iq, and colonies harboring the correct sequence (verified by restriction digest and complete DNA sequencing) were selected to express the respective S-COMT protein. Transformed DH5 α F'Iq cells were grown in modified TB medium containing ampicillin (100 μ g/ml) and kanamycin (25 μ g/ml). When the A₆₀₀ was between 0.4 and 0.6, cells were induced with 12 mM lactose and grown at 30°C for 16 h while shaking at 200 rpm. Cells were harvested by centrifugation at 5,000 \times g for 20 min, and spheroplasts were prepared by exposure to lysozyme. The spheroplasts were disrupted by sonication in 100 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 1 mM EDTA, 20% glycerol (v/v), 10 mM β -mercaptoethanol, 5 mM MgCl₂, and 10 μ M each of aprotinin, leupeptin, and pepstatin. The pellet obtained after centrifugation at 10,000 \times g for 20 min was discarded, and the supernatant was centrifuged overnight at 110,000 \times g. The resultant supernatant was applied to a pre-equilibrated nickel-nitrilo-triacetic acid column (1 ml resin per 50 nmol of enzyme). The column was washed with at least 50 column volumes of wash buffer [100 mM NaPO₄ (pH 8.0), 0.4 M NaCl, 20% glycerol (v/v), 10 mM β -mercaptoethanol, 5 mM MgCl₂, and 20 mM imidazole). The His-tagged protein was eluted with two column volumes of buffer (100 mM NaPO₄ (pH 7.4), 0.25 M NaCl, 20% glycerol (v/v), 10 mM β -mercaptoethanol, 5 mM MgCl₂, and 100 mM imidazole), and the eluate was dialyzed against dialysis buffer [100 mM NaPO₄ (pH 7.4), 0.25 M NaCl, 0.1 mM EDTA, 20% glycerol (v/v), 0.1 mM dithiothreitol, and 2 mM MgCl₂]. The purity of the protein was assessed by SDS-polyacrylamide gel electrophoresis and silver staining and by Western immunoblot using anti-COMT antibodies.

Selection of COMT-specific ScFv Antibodies from a Phage-Displayed Recombinant Antibody Library. A rodent phage-displayed recombinant antibody library (~2.9 \times 10⁹ members), generated by the Vanderbilt University Molecular Recognition Unit core facility, was used to obtain ScFv recombinant antibodies specific for COMT. All ScFv stemming from the recombinant antibody library had been cloned into *E. coli* TG1 cells using the pCANTAB5E phagemid vector (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Expressed ScFv display a tag recognized by the Pharmacia Anti-E

tag and HRP/Anti-E tag monoclonal antibodies. The Anti-E tag antibody can be used to detect ScFv bound to antigens in assays and can also be used to affinity-purify ScFv from bacterial extracts. Initial selections with purified His-COMT did not yield ScFv antibodies with sufficient affinity for use in immunoassays. Therefore, we attached another tag, GST using the plasmid pGEX-4T (Amersham Pharmacia Biotech, Inc.) to produce the recombinant purified fusion protein COMT-GST. Three rounds of phage antibody selection were performed using one ml of COMT-GST immobilized on Nunc Maxisorb tubes at 100 μ g of COMT-GST/ml PBS for the first round, 10 μ g/ml for the second round, and 1 μ g/ml for the third round of selection (28). Tubes and phage antibodies were blocked in 0.09-0.1% Tween 20 in PBS prior to selections. Phage antibodies were eluted from COMT-GST-coated tubes with 1 ml of 100 mM triethanolamine for the first two rounds of selection and with His-COMT at 10 μ g/ml PBS for the third round. Eluted phage antibodies were used to infect *E. coli* TG1 cells, which served as bacterial source for phage-displayed or soluble recombinant antibody production.

ICELISA to Determine ScFv Antigen Specificity. The ICELISA protocol, which accompanies Amersham Pharmacia's HRP/Anti-E tag conjugate, was used to detect and determine antigen specificity of ScFv produced by bacterial colonies. All assays were carried out in 384-well microtiter plates with individual wells either left uncoated or coated with 50 μ l of COMT-GST, His-COMT, or GST at 5 μ g/ml PBS.

Preparation and Purification of ScFv from Bacterial Periplasmic Extracts. Bacteria were grown overnight at 30°C in 250 ml of 2xYT medium with 100 μ g/ml ampicillin and 2% glucose with shaking at 100 rpm. Bacteria were centrifuged to pellet cells, resuspended in 2xYT medium with 100 μ g/ml ampicillin and 1 mM isopropyl- β -D-thiogalacto-pyranoside, incubated, and centrifuged as before. To prepare periplasmic extracts, bacterial pellets were resuspended sequentially in 10 ml of TES [0.2 M Tris-HCl (pH 8.0), 0.5 mM EDTA, and 0.5 M sucrose] and 15 ml of one-fifth TES [0.04 M Tris-HCl (pH 8.0), 0.1 mM EDTA, and 0.1 M sucrose] and placed on ice for 1 h or at -70°C until needed. Recombinant ScFv were purified from periplasmic extracts by affinity chromatography using an Amersham Pharmacia RPAS Purification Module according to the manufacturer's instructions.

Western Immunoblot of COMT. Purified recombinant His-COMT and COMT in breast cancer cell cytosol were resolved by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. Nitrocellulose filters were blocked for 1 h with 3% nonfat dry milk in PBS (3% NFD). The HRP/Anti-E tag conjugate was diluted 1:4,000 in 3% NFD, mixed with an equal volume ScFv in periplasmic extract, applied to COMT samples on nitrocellulose blots, and incubated for 1 h at room temperature. Blots were washed for 30 min in PBS containing 0.05% Tween 20 after which ScFv bound to COMT were visualized on film using an HRP-enhanced chemiluminescent substrate.

Competitive ICELISA to Quantify COMT. On the basis of preliminary assays, six bacterial clones produced ScFv that interacted with COMT-GST and His-COMT but not with GST. The ScFv bacterial clone designated C3 was selected based on optimal absorbance readings at 405 nm: 2.646 (COMT-GST), 2.702 (His-COMT), 0.136 (GST), and 0.208 (blank well). The competitive ICELISA was carried out at room temperature in a 384-well microtiter plate coated for 2 h with purified COMT-GST at 0.5 μ g/ml PBS, 50 μ l/well. Wells were emptied, filled with PBS containing 0.1% Tween 20 (PBST) and blocked for 15 min. Known concentrations of COMT-GST were mixed with C3-HRP/Anti-E immune complex (composed of purified C3, diluted to 2.7 μ g/ml, and HRP/Anti-E conjugate, diluted 1:8,000 in 3% NFD) to obtain a standard curve. Cytosol samples containing COMT were diluted 1:10 in C3-HRP/Anti-E immune complex. After a 90-min incubation, samples and COMT-GST standards were added in duplicate to the COMT-GST-coated microtiter wells, at 50 μ l/well. After a 1-h incubation, wells were washed seven times with PBS containing 0.05% Tween 20. Wells were tapped dry, and 50 μ l of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and hydrogen peroxide were added for color development and absorbance readings at 405 nm using a BIO-TEK ELx800NB plate reader (BIO-TEK Instruments, Inc., Winooski, VT). The plate reader's KCjr software was used to generate a standard curve, based on a four-parameter fit, and to calculate COMT concentrations in samples.

Assay of COMT Activity. Purified recombinant His-COMT (300 pmol) was reconstituted in 0.5 ml of 100 mM KPO₄ (pH 7.4), containing 5 mM MgCl₂, 10 mM β -mercaptoethanol, and 200 μ M SAM. Reactions were initiated by adding varying concentrations of each individual catechol estrogen (2, 3, 6,

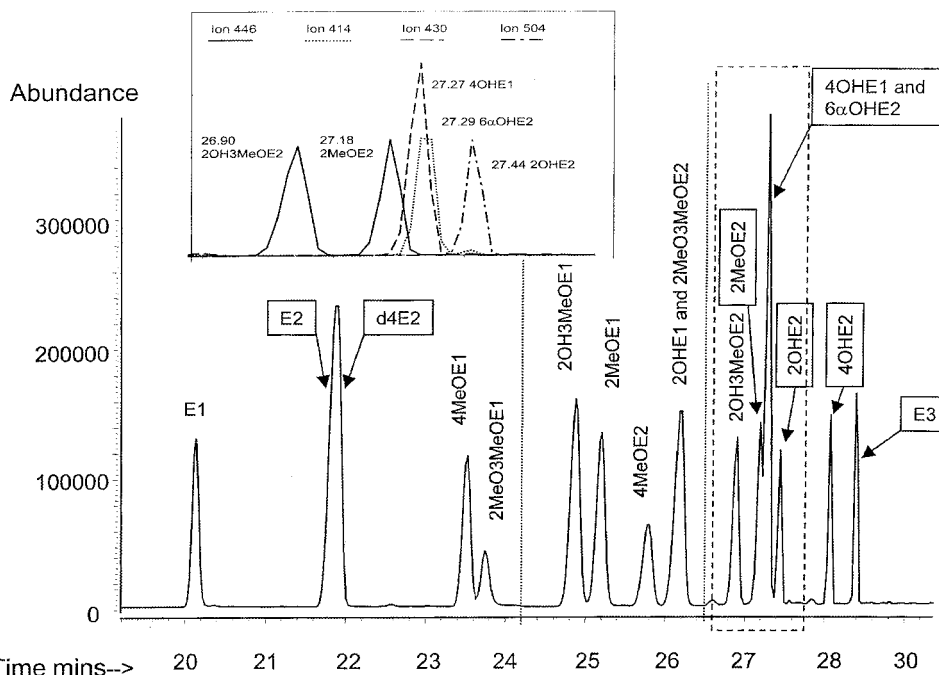


Fig. 1. Total ion chromatogram illustrating the separation of an equimolar mixture of estrogens, their metabolites, and the deuterated internal standard (d4E2). The vertical dotted lines indicate the position of the three different ion collection groups: 19–24.2 min [m/z 229, 257, 285, 287, 314, 315, 342, 343, 372, 373, 416, 417, and 420]; 24.2–26.5 min [m/z 257, 315, 342, 372, 373, 388, 389, 430, 431, 432, 446, and 447]; and 26.2–31 min [m/z 283, 309, 311, 315, 345, 373, 414, 430, 431, 446, 447, 504, and 505]. The inset shows the single-ion chromatograms (m/z 446, 414, 430, and 504) for the area within the dashed line on the total ion chromatogram where the peaks overlap. All compounds except 2-MeO-3-MeOE1 are chromatographed as TMS derivatives. The chromatography conditions are given in the text.

9, 12, 15, 20, 40, 60, 80, and 100 μM). Blanks contained all compounds except SAM. Reactions proceeded for 10 min at 37°C with gentle shaking and then were terminated by addition of 2 ml of CH_2Cl_2 . To determine COMT activity in breast cancer cells, ZR-75 and MCF-7 cells were harvested at confluency and homogenized in 100 mM KPO_4 (pH 7.4), 5 mM MgCl_2 , and 10 mM β -mercaptoethanol. After ultracentrifugation of the cell homogenate ($110,000 \times g$ for 30 min at 4°C), the supernatant cytosol was divided into aliquots for ICELISA, protein determination (BCA assay; Pierce, Rockford, IL), and COMT assay. The latter was carried out in the presence of 200 μM SAM and 100 μM catechol estrogen for 20 min at 37°C and then terminated by addition of CH_2Cl_2 . The concentration of endogenous catechol and methoxy estrogens was below the limit of detection by gas chromatography/mass spectrometry.

Thermal Inactivation. COMT thermal stability was measured as described by Scanlon *et al.* (29). Specifically, aliquots of recombinant wild-type and variant COMT were heated at 48°C for 15 min while control samples were kept on ice. The heated samples were returned to ice before measurement of enzyme activity. Thermal stabilities were expressed as H:C ratios, a commonly used measure of enzyme thermal stability (30, 31).

Extraction and Gas Chromatography/Mass Spectrometry Analysis of Catechol Estrogens. A deuterated internal standard (100 μl of 8 mg/l E2-d₄ in methanol) was added, and all estrogens were extracted into the CH_2Cl_2 by vortex mixing for 30 s. The CH_2Cl_2 fraction (1.5 ml) was evaporated to dryness under air, and volatile TMS derivatives were prepared by heating the residue with 100 μl of 50% NO-bis(trimethylsilyl)trifluoroacetamide/1% trimethyl chlorosilane in acetonitrile at 56°C for 30 min. The TMS derivatives of the estrogen metabolites were separated by gas chromatography (H-P 5890, Hewlett-Packard, Wilmington, DE) on a 5% phenyl methyl silicone stationary phase fused silica capillary column (30 m \times 0.2 mm \times 0.5 μm film, HP5; Hewlett-Packard). Helium carrier gas was used at a flow of 1 ml/min. The injector was operated at 250°C, with 2 μl injected in the splitless mode, with a purge (60 ml/min helium) time of 0.6 min. The oven temperature was held at 180°C for 0.5 min, then raised at 6°C/min to 250°C where it was held for 17 min, then raised to 300°C at 8°C/min to give a total run time of 35.42 min. This program permitted adequate separation of a wide range of estrogen metabolites. Retention times (in min) for the TMS derivatives were: E1, 20.13; E2 and E2-d₄, 21.89; 4-MeOE1, 23.52; 2-MeO-3-MeOE1 (underivatized), 23.75; 2-OH-3-MeOE1, 24.87; 2-MeOE1, 25.2; 4-MeOE2, 25.78; 2-OHE1 and 2-MeO-3-MeOE2, 26.19; 2-OH-3-MeOE2, 26.9; 2-MeOE2, 27.18; 4-OHE1, 27.27; 6 α -OHE2, 27.29; 2-OHE2, 27.44; 4-OHE2, 28.06; and E3, 28.38. The EI mass spectrometer (H-P 5970) was operated in the selected ion

monitoring mode from 18 to 30 min. Ions monitored were TMS-E1, 342, 257, and 343; TMS-E2-d₄, 420, 421, and 287; TMS-E2, 416, 417, and 285; TMS-4-MeOE1, TMS-2-OH-3-MeOE1, TMS-2-MeOE1, and TMS-3-MeO-4-OHE1, 372, 373, and 342; 2-MeO-3-MeOE1, 314, 315, and 229; TMS₂-4-MeOE2, TMS₂-2-OH-3-MeOE2, TMS₂-2-MeOE2, and TMS₂-3-MeO-4-OHE2, 446, 447, and 315; TMS₂-2-OHE1 430, 431, and 432; TMS-2-MeO-3-MeOE2, 388, 389, and 257; TMS₂-4-OHE1, 430, 431, and 345; TMS₂-6 α -OHE2, 414, 283, and 309; TMS₃-2-OHE2 and TMS₃-4-OHE2, 504, 505, and 373; and TMS₃-E3, 504, 505, and 311 (Fig. 1). The instrument was calibrated by simultaneous preparation of an 11-point calibration over the range 0–22 nmol/tube of each compound. Sensitivity was determined to be between 0.02 and 0.04 nmol/tube (400–800 fmol on column) for the various compounds. Preparation of the TMS derivatives improved chromatography and sensitivity significantly. Derivatization was performed at 56°C because use of a higher temperature resulted in the loss of some estrogen derivatives (particularly the 2-OH metabolite of estrone). Derivatization was demonstrated to be complete at 20 min, as evidenced by the absence of detectable amounts of underivatized estrogens in the highest calibrator when the detector was operated in full scan mode. Absolute extraction efficiency for E2, 2-OH-E2, and 4-OH-E2 at 3.5 nmol/tube was 119, 96, and 107%, assessed by comparison with injections of spiked solvent samples onto the gas chromatograph. Internal standard added prior to extraction compensated for deviation from 100% recovery for all investigated compounds.

Statistical Analysis. Kinetic parameters (K_m and k_{cat}) for the enzyme reactions were determined by nonlinear regression analysis using the computer program GraphPad Prism (San Diego, CA).

RESULTS

We performed PCR and restriction endonuclease digestion to identify the wild-type and variant *COMT* alleles. We introduced a *Bsp*HI restriction site into the C1 primer (see “Materials and Methods”; underlined nucleotide) to reveal the methionine allele in codon 108 of the *COMT* gene. *Bsp*HI is a 6-base cutter with a single recognition site on the PCR product of the methionine allele and no site on the valine allele. In contrast, the 4-base cutter *Nla*III used by Lachman *et al.* (11) cleaves three sites on the methionine allele and two sites on the valine allele, yielding relatively small restriction fragments of 67 and 71 bp, which are not easily distinguished from each other. Digestion of the *COMT* PCR product with *Bsp*HI yielded bands of 160 bp for the

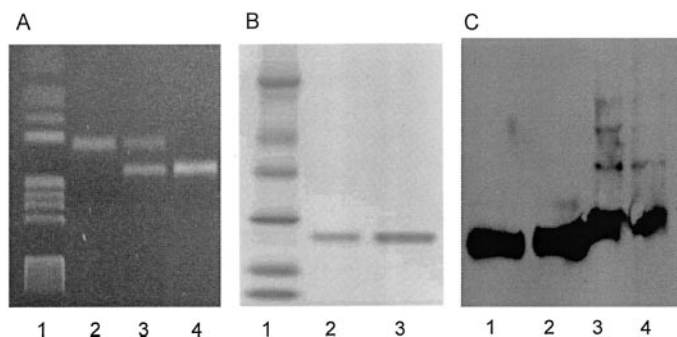


Fig. 2. Analysis of *COMT* gene and protein. A, Analysis of *COMT* genotypes by PCR amplification and digestion with *Bsp*HI, followed by agarose gel electrophoresis, shows bands of 160 bp for the *Val/Val* genotype (Lane 2); 160, 125, and 35 bp for the *Val/Met* genotype (Lane 3); and 125 and 35 bp for the *Met/Met* genotype (Lane 4). The small 35-bp fragment is not visualized on this low melting agarose gel. Lane 1, the molecular size marker. B, SDS-PAGE of purified wild-type and variant *COMT* subjected to silver stain shows wild-type (Lane 2) and variant (Lane 3) *COMT*. Lane 1 contains the molecular weight marker. C, Western immunoblot using anti-*COMT* antibody H6 shows recombinant wild-type *COMT* (Lane 1), recombinant variant *COMT* (Lane 2), wild-type *COMT* in ZR-75 cytosol (Lane 3), and variant *COMT* in MCF-7 cytosol (Lane 4).

Val/Val genotype; 160, 125, and 35 bp for the *Val/Met* genotype; and 125 and 35 bp for the *Met/Met* genotype (Fig. 2A). Breast cancer cell lines ZR-75 (*Val/Val*) and MCF-7 (*Met/Met*) served as a source for wild-type and variant S-*COMT* cDNA, respectively. His-tagged wild-type and variant S-*COMT*s were expressed and purified by nickel-nitrilo-triacetic acid chromatography. Each recombinant protein was electrophoretically homogeneous as judged by SDS-PAGE and silver staining, which revealed a single band at M_r 25,000 (Fig. 2B). We developed *COMT*-specific ScFv antibodies to further characterize the recombinant *COMT* and to demonstrate the presence of wild-type and variant *COMT* in breast cancer cell lines ZR-75 and MCF-7, respectively. Initial attempts to select for phage-displayed, *COMT*-specific ScFv using purified His-*COMT* yielded antibodies whose affinity was too low for use in immunoassays. Therefore, we prepared recombinant, purified *COMT*-GST to generate antibodies with greater affinity. The ScFv bacterial clone designated H6 proved optimal, yielding the following ICELISA absorbance readings: 2.551 (*COMT*-GST), 0.441 (His-*COMT*), 0.141 (GST), and 0.151 (blank well). The Western immunoblot using anti-*COMT* antibody H6 showed one major

band at M_r 25,000 for recombinant wild-type and variant *COMT* (Fig. 2C, Lanes 1 and 2). Similarly, wild-type and variant *COMT* in cytosol of ZR-75 and MCF-7 cells, respectively, migrated predominantly as one band (Fig. 2C, Lanes 3 and 4). However, the cytosol protein migrated slightly higher than the recombinant protein, probably because of posttranslational modification.

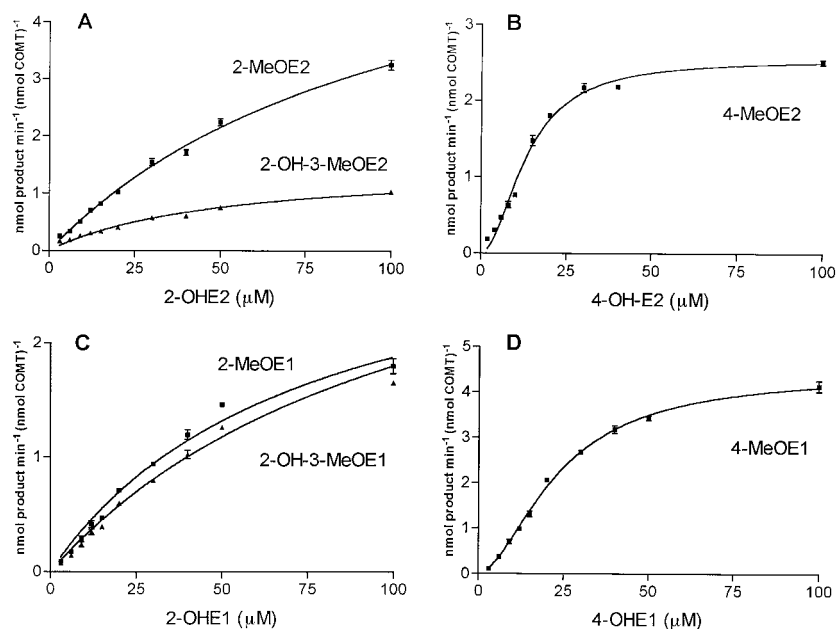
COMT activity was assessed by determining the methylation of the substrates 2-OHE2, 4-OHE2, 2-OHE1, and 4-OHE1 (Fig. 3). The reaction kinetics were determined in two replicate experiments at 10 different concentrations of each substrate. The resulting K_m and k_{cat} are presented in Table 1. *COMT* catalyzed the formation of mono-methyl ethers at 2-OH, 3-OH, and 4-OH groups. Dimethyl ethers were not observed. In the case of 2-OHE2 and 2-OHE1, methylation occurred at 2-OH and 3-OH groups, resulting in the formation of 2-MeOE2 and 2-OH-3-MeOE2, and 2-MeOE1 and 2-OH-3-MeOE1, respectively. In contrast, in the case of 4-OHE2 and 4-OHE1, methylation occurred only at the 4-OH group, resulting in the formation of 4-MeOE2 and 4-MeOE1, respectively. 3-MeO-4-OHE2 and 3-MeO-4-OHE1 were not observed. As shown in Fig. 3, the rates of methylation of 2-OHE2 and 2-OHE1 yielded typical hyperbolic patterns, whereas 4-OHE2 and 4-OHE1 exhibited a sigmoid curve pattern. Overall, *COMT* displayed the highest catalytic efficiencies k_{cat}/K_m in the formation of 4-MeO products (142 and 126 $\text{mm}^{-1}\text{min}^{-1}$), followed by the 2-MeO products (63 and 45 $\text{mm}^{-1}\text{min}^{-1}$), and lastly the 3-MeO products (29 and 38 $\text{mm}^{-1}\text{min}^{-1}$; Table 1). Competition experiments using an equimolar concentration of all four catechol estrogens revealed the following order of product formation: 4-MeOE2 > 4-MeOE1 \gg 2-MeOE2 > 2-MeOE1 > 2-OH-3-MeOE1 > 2-OH-3-MeOE2 (Fig. 4). The experimental conditions

Table 1 Kinetic parameters for *COMT*-mediated catechol estrogen metabolism

Products	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{mm}^{-1}\text{min}^{-1}$)	Hill coefficient
2-MeOE2	108 \pm 9	6.8 \pm 0.4	63 \pm 6	NA ^a
2-OH-3-MeOE2	51 \pm 5	1.5 \pm 0.1	29 \pm 3	NA
4-MeOE2	24 \pm 3	3.4 \pm 0.2	142 \pm 20	1.6 \pm 0.2
2-MeOE1	74 \pm 8	3.3 \pm 0.2	45 \pm 6	NA
2-OH-3-MeOE1	73 \pm 16	2.8 \pm 0.4	38 \pm 10	NA
4-MeOE1	53 \pm 6	6.7 \pm 0.4	126 \pm 16	2.0 \pm 0.4

^a NA, not applicable; the best fit was to a Michaelis-Menten curve.

Fig. 3. Determination of kinetic parameters of *COMT*-mediated metabolism of individual catechol estrogens 2-OHE2 (A), 4-OHE2 (B), 2-OHE1 (C), and 4-OHE1 (D). Data are represented as means of two replicate assays; bars, SD. The points were fitted using nonlinear regression with the computer program GraphPad PRISM (San Diego, CA). The data reflect the best fit (judged by P) according to a comparison of Michaelis-Menten and sigmoidal equations. The equations used were: Michaelis-Menten, $v = (V_{max} S)/(K_m + S)$; sigmoidal, $v = (V_{max} S^n)/(K_m^n + S^n)$.



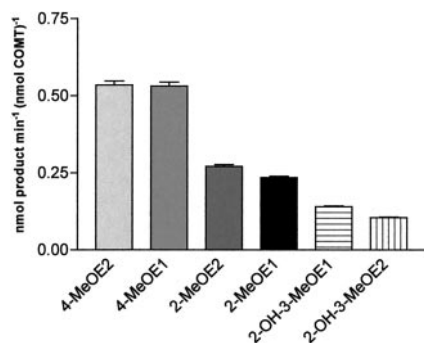


Fig. 4. Competitive COMT methylation of equimolar concentration (5 μM) of 2-OHE2, 4-OHE2, 2-OHE1, and 4-OHE1. Data are represented as means ($n = 3$); bars, SD.

used for the enzyme reaction (10 min at 37°C) did not show a difference in recombinant wild-type and variant COMT activities. However, heat inactivation (15 min at 48°C) prior to the enzyme reaction revealed a difference in thermal stability expressed as H:C ratio between wild-type and variant COMT. As shown in Fig. 5, the H:C ratio of the variant enzyme was significantly lower than the ratio of the wild-type enzyme, leading to 2–3-fold lower levels of product formation after heating.

To directly compare the enzymatic activities of wild-type COMT in ZR-75 cells and variant COMT in MCF-7 cells, we developed an ICELISA to quantify both enzymes as proteins. The H6 antibody, which we used for Western immunoblot, proved to be suboptimal for ICELISA. Therefore, we selected another ScFv antibody, designated C3, based on absorbance readings and an optimal dose-response curve for the concentration range 2.5–2500 ng/ml (Fig. 6). Wild-type and variant COMT were indistinguishable by ICELISA. The concentration of COMT in ZR-75 and MCF-7 breast cancer cells was similar, *i.e.*, 7.9 ± 1.1 and 8.1 ± 1.5 $\mu\text{g}/\text{mg}$ cytosol protein. However, the enzymatic activity with respect to catechol estrogens differed significantly, as shown in Fig. 7. The variant COMT isoform in MCF-7 cells produced 2–3-fold lower product levels than wild-type COMT in ZR-75 cells.

DISCUSSION

COMT catalyzes the methylation of both 2-OH and 4-OH catechol estrogens to methoxy estrogens. However, our data reveal major differences in the interaction of 2-OHE and 4-OHE substrates with the enzyme. Methylation of 2-OHE substrates exhibits Michaelis-Menten saturation kinetics and yields two products, *i.e.*, 2-methoxy- and 3-methoxyestrogens. In contrast, the methylation of 4-OHE substrates displays sigmoid saturation kinetics indicating cooperative binding and yields only a single product, *i.e.*, 4-methoxyestrogen. COMT-mediated methylation of 4-OHE2 and 4-OHE1 has been proposed as a pathway for the formation of 3-MeO-4-OHE2 and 3-MeO-4-OHE1, respectively (26). Our results show that 3-MeO-4-OHE2 and 3-MeO-4-OHE1 are not formed by COMT. The main structural difference

between 2-OH and 4-OH catechol estrogens is the proximity of the 4-OH group to the B ring of the steroid. Clearly, the 2-OH and 3-OH groups in 2-OHE appear to be similar in reactivity, whereas the 3-OH and 4-OH groups in 4-OHE differ in reactivity to the point that in the latter only the 4-OH group becomes methylated. The difference in regioselective reactivity is not limited to the catechol estrogens but extends to the corresponding quinones. For example, the 2,3-quinone formed from 2-OHE has a half-life of 42 at pH 7.4 and 37°C, whereas the 3,4-quinone formed from 4-OHE has a half-life of 12 min under identical conditions (32). Reactions of 4-methylimidazole with 2,3-estrone quinone and 3,4-estrone quinone also showed greater reactivity and decreased stability of the former (33).

Competitive COMT-catalyzed methylation of equimolar concentrations of 2-OHE2, 4-OHE2, 2-OHE1, and 4-OHE1 yielded preferential formation of 4-MeO products, followed by 2-MeO and 3-MeO products. This order matches that predicted by the experiments with individual catechol estrogens, in which COMT displayed the highest catalytic efficiencies for the formation of 4-MeO products, followed by 2-MeO and 3-MeO products. The difference in regioselective reactivity of 2-OH and 4-OH catechol estrogens and their respective quinones translates into biological differences with respect to carcinogenicity. Treatment with 4-OHE2 and 4-OHE1, but not 2-OHE2 and 2-OHE1, induced renal cancer in Syrian hamster (34, 35). Analysis of renal DNA demonstrated that 4-OHE2 and 4-OHE1 significantly increased DNA adduct (8-hydroxyguanosine) levels, whereas the corresponding 2-OH catechol estrogens had no effect (36). Similarly, 4-OHE2 induced DNA single-strand breaks whereas 2-OHE2 had a negligible effect (37). Experiments with calf thymus DNA showed that 2,3-quinones preferentially form stable adducts, whereas 3,4-quinones are more likely to produce depurinating adducts (17, 38). When both stable and depurinating adducts were considered, the 3,4-quinones produced a much higher level of binding with DNA (39).

The regioselective methylation of 2-OH and 4-OH catechol estrogens by COMT influences not only the level of potential DNA damage but also the concentration of 2-MeOEt, an antiproliferative

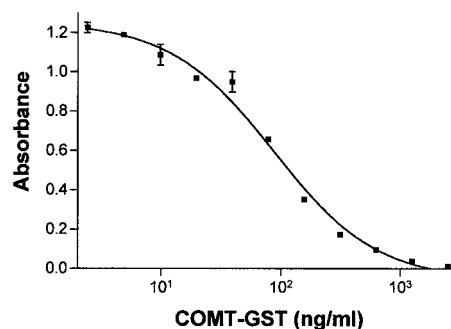


Fig. 6. ICELISA dose-response curve using COMT-GST standards over a range of 2.5–2500 ng/ml ($R^2 = 0.99$). Data represent means of duplicate readings; bars, SD. The concentration of COMT in samples was obtained by correcting for the molecular weight contribution of GST (M_r 26,000) in the COMT-GST fusion protein (M_r 51,000).

Fig. 5. Comparison of thermal stability of wild-type and variant COMT activity. Products formed by methylation of 2-OHE2 and 4-OHE2 are shown in A, and the corresponding products formed from 2-OHE1 and 4-OHE1 are shown in B. Data are represented as means ($n = 3$); bars, SD.

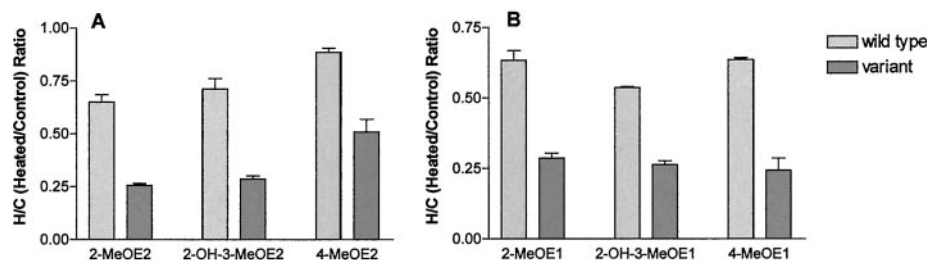
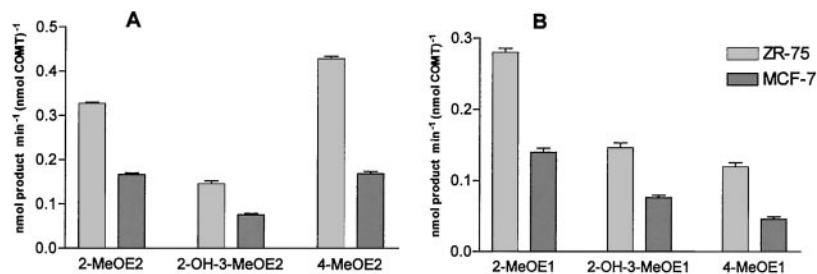


Fig. 7. Comparison of wild-type COMT activity in ZR-75 cells and variant COMT activity in MCF-7 cells. Products formed by methylation of 2-OHE2 and 4-OHE2 are shown in A, and the corresponding products formed from 2-OHE1 and 4-OHE1 are shown in B. Data represent means ($n = 3$); bars, SD.



metabolite for human breast cancer, lung cancer, and leukemia cells (19, 20, 23, 24). The antiproliferative effect of 2-MeOE2 appears to be concentration dependent and involve several mechanisms. At nano- and micromolar concentrations, 2-MeOE2 caused disruption of microtubule function, induction of apoptosis, and inhibition of angiogenesis (21–24). At millimolar concentrations, 2-MeOE2 caused chromosome breaks and aneuploidy (40). Comparison of 2-MeOE2 and 4-MeOE2 showed no antiproliferative action of the latter compound, suggesting that 2-MeOE2 may be the only endogenous estrogen metabolite that inhibits mammary carcinogenesis (26). However, the second *O*-methylation product of 2-OHE2, 2-OH-3-MeOE2, has not been examined, and a systematic comparison of 2-MeOE2 and 2-MeOE1 has not yet been performed.

S-COMT consists of eight α -helices and seven β -strands (41). Residue 108Val, which is located in the turn between $\alpha 5$ and $\beta 3$, is not part of the coenzyme (SAM), Mg^{2+} ion, or substrate binding sites. Although not directly involved in the methyl transfer reaction, residue 108 appears critically important for overall configuration and stability of the enzyme. The substitution 108Val \rightarrow Met changes the thermotropic behavior of COMT. Using the H:C ratio as a function of thermal stability and dopamine as a substrate, Lotta *et al.* (31) observed an 80% reduction in enzyme activity when variant COMT, expressed in *E. coli*, was incubated for 15 min at 40°C, whereas the wild-type COMT activity remained stable. Our reaction conditions differed from those of Lotta *et al.* (31) in that we used catechol estrogens as substrates and purified recombinant COMT. Nevertheless, the H:C ratio of the variant enzyme was significantly lower than the ratio of the wild-type enzyme, leading to 2–3-fold lower levels of product formation. These findings indicate that the substitution 108Val \rightarrow Met renders COMT more thermolabile and thereby lowers enzyme efficiency toward all substrates.

Using 3,4-dihydroxybenzoic acid as substrate, Syvanen *et al.* (12) showed that COMT activity in RBCs from individuals with the homozygous *Met/Met* genotype was $\sim 60\%$ lower than that of homozygous *Val/Val* individuals, *i.e.*, 0.21–0.43 versus 0.55–1.03 pmol \cdot min⁻¹ \cdot mg⁻¹ protein. In this study, we used catechol estrogens as physiological substrates and developed an ICELISA to directly measure the COMT concentration in breast cancer cells. Although ZR-75 and MCF-7 contain similar amounts of COMT, they differ in genotype and enzymatic activity. The catalytic activity of variant COMT in MCF-7 cells was 2–3-fold lower than that of wild-type COMT in ZR-75 cells. Because COMT is expressed ubiquitously, it appears that the *COMT* genotype significantly affects levels of catecholamines and catechol estrogens throughout the body. This means that tissue concentrations of catechol estrogens will differ significantly during the reproductive life of women with variant COMT compared with women with wild-type *COMT* genotype. Therefore, the exposure to 2-MeOE2 and its purported beneficial effect will be considerably less in women with variant COMT.

In contrast to COMT, the cytochrome P-450 (CYP) enzymes, CYP1A1 and CYP1B1, are expressed in a tissue-specific manner, with high levels in breast and low levels in liver (42, 43). With regard

to estrogens, the multifunctional CYP1A1 and CYP1B1 catalyze two sequential reactions in the human breast, *i.e.*, the hydroxylation of E2 and E1 to their respective catechol estrogens, followed by the oxidation of catechol estrogens to estrogen quinones (44, 45). Thus, CYP1A1 and CYP1B1 generate catechol estrogen substrates for COMT, and at the same time, they compete with COMT by converting the catechol estrogens to estrogen quinones. To complicate matters further, CYP1A1 and CYP1B1 differ in catalytic efficiency as estrogen hydroxylases and in their principal site of catalysis with regard to the A ring of the steroid (46–48). CYP1B1 exceeds CYP1A1 in its catalytic efficiency as an estrogen hydroxylase and has its primary activity at the C-4 position, whereas CYP1A1 has its primary activity at the C-2 position in preference to 4-hydroxylation. Thus, CYP1B1 produces mostly 4-OHE2 and 4-OHE1, which are methylated faster by COMT than 2-OHE2 and 2-OHE1, the principal products of CYP1A1. Finally, genetic variants of each of these three enzymes have been identified, some with proven or suspected change in catalytic activity (27, 49–52). For example, the CYP1B1 119Ser variant is about three times more active in converting E2 to 4-OHE2 than wild-type CYP1B1 119Ala (27). In this study, we determined that the COMT 108Met variant is about three times less active in methylating 4-OHE2 to 4-MeOE2 than wild-type COMT 108Val. Thus, a hypothetical woman A with the wild-type genotype combination CYP1B1 (119Ala/Ala), COMT (108Val/Val) is likely to be exposed to significantly lower 4-OHE2 levels than woman B with the variant genotype combination CYP1B1 (119Ser/Ser), COMT (108Met/Met). In view of the potential genotoxic effect of 4-OH catechol estrogens and the lifelong effect of variant genotypes on reaction rates, one would predict that woman B had a higher risk of developing breast cancer than woman A. Of course, these genetic risk factors will be modified by traditional, nongenetic risk factors such as age, parity, menopausal status, and body mass index.

In summary, we have defined the role of COMT in catechol estrogen metabolism and determined the difference in catalytic efficiency between wild-type and variant COMT. To prove the negative impact of variant COMT on breast cancer risk will require consideration of other enzymes participating in mammary estrogen metabolism as well as of traditional risk factors in large-scale molecular epidemiological studies.

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Catechol-O-Methyltransferase (COMT)-mediated Metabolism of Catechol Estrogens: Comparison of Wild-Type and Variant COMT Isoforms

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