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Activation of Peroxisome Proliferator-activated Receptor α by Substituted Urea-derived Soluble Epoxide Hydrolase Inhibitors

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Running title: sEH inhibitors activate PPARα

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List of nonstandard abbreviation: CUDA, *N*-cyclohexyl-*N'*-dodecanoic acid urea; AUDA, *N*-adamantanyl-*N'*-dodecanoic acid urea; EETs, epoxyeicosatrienoic acids; sEH, soluble epoxide hydrolase; DHETs, dihydroxyeicosatrienoic acids; DCU, *N,N'*-dicylclohexylurea; CDU, *N*-cyclohexyl-*N'*-dodecylurea; PPAR, peroxisome proliferator-activated receptor; CPT1A, carnitine palmitoyltransferase 1A; GW7647, 2-(4-(2-(1-cyclohexanebutyl-3-cyclohexylureido)ethyl)phenylthio)-2-methylpropionic acid; Wy-14643, pirinixic acid; CUOA, *N*-cyclohexyl-*N'*-octanoic acid urea; CUHA, *N*-cyclohexyl-*N'*-hexanoic acid urea.

ABSTRACT

Soluble epoxide hydrolase (sEH) plays a major role in regulating vascular epoxyeicosatrienoic acid metabolism and function, and substituted urea derivatives that inhibit sEH activity reduce blood pressure in hypertensive rats. We found that substituted urea derivatives containing a dodecanoic acid group, besides effectively inhibiting sEH, increased peroxisome proliferator-activated receptor (PPAR) α activity. In PPARα transfected COS-7 cells, treatment with 10 μM *N*-cyclohexyl-*N'*-dodecanoic acid urea (CUDA) or *N*-adamantanyl-*N'*-dodecanoic acid urea (AUDA) produced 6-fold and 3-fold increases, respectively, in PPARα activation. Neither CUDA nor AUDA activated PPARδ or PPARγ directly, indicating selectivity for PPARα. CUDA did not alter PPARα protein expression, and it competitively inhibited the binding of Wy-14643 to the ligand binding domain of PPARα, suggesting that it functions as a PPARα ligand. CUDA and AUDA were metabolized to chain-shortened β-oxidation products, a process that reduced their potency as sEH inhibitors and their ability to bind and activate PPARα. *N,N'*-dicylclohexylurea and *N*-cycloxyl-*N'*-dodecylurea, sEH inhibitors that do not contain a carboxylic acid group, did not activate PPARα. In HepG2 cells, CUDA increased the expression of the PPARα responsive gene carnitine palmitoyltransferase 1A. We conclude that CUDA and AUDA, by virtue of their carboxylic acid substitution, activate PPARα in addition to potently inhibiting sEH. Further development of these compounds could lead to a class of agents with hypotensive and lipid lowering properties that may be valuable for the prevention and treatment of cardiovascular disease.

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INTRODUCTION

Epoxyeicosatrienoic acids (EETs) are endogenous lipid mediators synthesized from arachidonic acid by cytochrome P450 epoxygenases that play a broad role in the regulation of cardiovascular function (Capdevila et al., 2000; Zeldin 2001; Roman, 2002; Spector et al., 2004). EETs have been identified as endothelium-derived hyperpolarizing factors in coronary, renal and internal mammary vessels. They are rapidly incorporated into phospholipids, a process that may modulate their diverse cellular actions including regulation of tyrosine kinase, mitogen-activated protein kinase, extracellular signal regulated kinases 1 and 2, cyclooxygenase, Ca^{2+} mobilization, $G_s\alpha$ protein signaling pathways, and expression of adhesion molecules (Spector et al., 2004). The predominant metabolic pathway for EETs is conversion to the corresponding dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH), and inhibitors of sEH block this conversion in vascular cells (Weintraub et al., 1999; Fang et al., 2001). This increases the cellular incorporation and retention of EETs, enhances the flux of EETs into alternative β-oxidation and chain-elongation metabolic pathways, and potentiates endothelium-dependent relaxation (Weintraub et al., 1999; Fang et al., 2001).

Disruption of the sEH gene in male mice decreases blood pressure (Sinal et al., 2000), suggesting that sEH could be a novel target for the treatment of hypertension. This finding has generated considerable interest in developing effective sEH inhibitors, and derivatives of urea that are potent, selective and stable sEH inhibitors recently have been synthesized (Morisseau et al., 1999). *N*,*N'*-dicylclohexylurea (DCU) is a representative compound of this class. Treatment with DCU reduced blood pressure in spontaneously hypertensive rats (Yu et al., 2000), and related compounds inhibited the proliferation of cultured vascular smooth muscle cells (Davis et al., 2002). *N*-Cyclohexyl-*N'*-dodecylurea (CDU), in which a hydrocarbon chain replaces the *N'*-

cyclohexyl group of DCU, increases the potency of sEH inhibition 8- to 16-fold. However, the potential pharmacological usefulness of CDU is limited by its low aqueous solubility. Therefore, the compound was structurally modified to produce more H_2O-soluble derivatives. Two of these derivatives that contain a carboxylic acid substitution, *N*-cyclohexyl-*N'*-dodecanoic acid urea (CUDA) and *N*-adamantidyl-*N'*-dodecanoic acid urea (AUDA), increase H_2O solubility without an appreciable reduction in the potency of sEH inhibition (Morisseau et al., 2002).

Peroxisome proliferator-activated receptors (PPAR) are members of the nuclear steroid hormone receptor superfamily that function to transduce a variety of nutritional and inflammatory signals. PPARα, a member of this class, activates the expression of genes that regulate lipid metabolism. Many hypolipidemic drugs, polyunsaturated fatty acids and eicosanoids activate PPARα (Yu et al., 1995; Forman et al., 1997; Kliewer et al., 1997; Bishop-Bailey et al., 2003), and 14,15-EET and its hydroxylated derivatives recently were shown to also activate PPARα (Cowart et al., 2002). Because sEH inhibition substantially increases the intracellular accumulation and retention of 14,15-EET (Fang et al., 2001), we investigated whether this might produce an EET-mediated activation of PPARα.

All of the urea-derived selective sEH inhibitors that we tested had similar inhibitory effects on the conversion of 14,15-EET to DHET. However, we unexpectedly found that only the sEH inhibitors containing a *N'*-carboxylic acid substitution activated PPARα, indicating that the activation occurred through an EET-independent mechanism. This report describes the activation of PPARα by CUDA and AUDA in a transfected COS-7 cell model system, and provides evidence that CUDA functions as a PPARα ligand. The metabolism of CUDA and AUDA also was investigated in the COS-7 cells to determine whether this might affect the ability of these sEH inhibitors to function as PPARα ligands.

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METHODS

Cell Culture ---- COS-7 cells were purchased from ATCC and suspended in Dulbecco's Minimum Essential Medium (DMEM) supplemented with Minimum Essential Medium (MEM) nonessential amino acids, MEM vitamin solution, 15 mmol/L HEPES, 2 mmol/L L-glutamine, 50 µmol/L gentamicin, and 10% fetal bovine serum (FBS). The suspended cells were counted with a hemocytometer and plated into 25 cm^2 flasks at the density of 4 x 10^4 cells/ml, and the cultures were maintained until confluent at 37°C in a humidified atmosphere containing 5% CO_2. Stocks were subcultured weekly by trypsinization, and the cells were transferred into 6-well plates for all experiments. Cultures were used between passage numbers 12 and 25.

Transient Transfection of COS-7 Cells---- PPARα was overexpressed in COS-7 cells, which inherently have very low or absent expression of this gene (Wurch et al., 2002). For comparison, additional COS-7 cultures were transfected with the PPARδ or PPARγ genes. The plasmids containing cDNA for mouse PPARα, PPARδ, PPARγ, and the PPAR-responsive luciferase reporter construct were kindly provided by Dr. Ronald M. Evans (Salk Institute) (Forman et al., 1995; Forman et al., 1997; Wang et al., 2003). Briefly, these expression vectors contained the cytomegalovirus IE promoter/enhancer (pCMX) upstream of either wild-type mouse PPARα (pCMX-mPPARα), mouse PPARδ (pCMX-mPPARδ), or mouse PPARγ1 (pCMX-mPPARγ) genes. The plasmids were further replicated and purified using QIAprep Miniprep (Qiagen Inc.), and they were analyzed by restriction digest and agarose gel electrophresis.

COS-7 cells (60-70% confluent) in 60 mm dishes were transiently transfected using SuperFect with 0.02 µg of PPARα, δ, or γ; 0.02 µg of the PPAR-responsive-luciferase (tk-PPREx3-luc) reporter construct, and 0.2 µg of a ß-galactosidase (β-Gal)-expression plasmid. The

β-Gal plasmid was used as an internal control to normalize for transfection efficiency. Following incubation for 24 h, the medium containing the plasmids was removed and the cultures incubated with various concentrations of sEH inhibitors for 18 h. These inhibitors were dissolved in dimethyl sulfoxide (DMSO); the final concentration of DMSO in the mediun was 0.1% (v/v). After the cells were collected and lysed, luciferase and ß-Gal activities were measured (Chen et al., 2005; Zhu et al., 2001), and the luciferase activity was normalized to the ß-Gal activity.

Western Blot Analysis -- Cells were placed in an ice-bath and lysed with three 20 s bursts of sonic irradiation (Tekar Sonic Disruptor). The protein content of the cell lysate was measured by the Bradford method (Bradford, 1976), using a Bio-Rad Protein Assay kit. Samples were denatured with a sodium dodecylsulfate (SDS) loading buffer at 95° C for 5 min, and the proteins were separated in a SDS-10% polyacrylamide gel with a 5% stacking gel in SDS-Tris-glycine running buffer. The proteins were transferred electrophoretically to a nitrocellulose membrane, which was then blocked with 5% (w/v) non-fat milk in 0.02 M Tris/0.15 M NaCl buffer, pH 7.45, with 0.1% Tween 20 (TTBS) for 1 h. After an overnight incubation in TTBS buffer containing specific rabbit anti-serum raised against a peptide corresponding to amino acid 22-36 of human/murine/rat PPARα (1:1000, Cayman Chemical), the blot was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:10000, Boehringer Mannheim Corp.) for 1 h at room temperature, and the anti-PPARα antibodies were detected using an ECL detection system (Pierce Chemical Co.) and exposure to X-ray film. Following this, the membrane was stripped and re-probed with antibody against ß-actin (Sigma) as described above, and the density of the β-actin band was used to normalize for protein loading.

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Carnitine Palmitoyltransferase 1A (CPT1A) mRNA Analysis by real time RT-PCR ------ Total RNA from cultures was isolated with TRIzol reagent (Life Technologies, Grand Island, NY, U.S.A.), and the RNA content was measured spectrophotometrically (Fang et al, 2000b). Two µg of total RNA from each sample was reversed transcribed. The resulting cDNAs were diluted (1/10-1/50) and equal amounts were aliquoted for real time PCR analysis using a Stratogene Mx 3000P instrument. Primers and FAM-labeled probes for CPT1A, GAPDH (house-keeping gene) and Universal Taqman$^{@}$ master mix were purchased from Applied Biosystems, Inc. (ABI) (Foster City, Ca). CPT1A mRNA was assayed by the comparative quantitation method and the calculated differences in mRNA expression were determined according to User Bulletin 2 (10/2001, ABI systems). Gene expression data are expressed as fold differences from the control cells and have been normalized to the expression of GAPDH.

Binding competition assays ---- [^{3}H]pirinixic acid ([^{3}H]Wy-14643), 7.5 Ci/mmol, (American Radiolabeled Chemicals, St. Louis, MO), together with CUDA or CUDA metabolites were incubated with recombinant PPARα ligand binding domain (GST-mPPARα-LBD) in a buffer containing 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM DTT, and 200 ng/ml ovalbumin (Forman et al., 1997). After incubated at 25°C for 30 min and chilling on ice for 15 min, the free and bound ligands were separated by Sephadex G-25 (Sigma) columns in a buffer containing 15% glycerol, 25 mM Tris-HCl, pH 7.8, 0.05% Triton X-100, 0.5 mM EDTA, and 75 mM KCl. The quantity of bound ligands was determined by liquid scintillation counting.

EET Metabolism ---- COS-7 cells were incubated with sEH inhibitors in modified DMEM for 30 min, after which 1 µM [^{3}H]14,15-EET was added and the incubation continued in the presence of the inhibitor. After 1 h, the medium was collected, and the cells were washed twice

with cold phosphate-buffered saline solution and harvested by scraping into methanol. Radioactivity in an aliquot of the medium and cell lipid extract was measured by liquid scintillation counting. The remainder of the medium was extracted twice with 4 ml of ice-cold ethyl acetate, and after the extracts were combined, the solvent was evaporated under N_2 and the lipid residue dissolved in acetonitrile. The lipids were separated by reverse-phase high performance liquid chromatography (HPLC), and the column effluent was mixed with scintillator solution and passed through an in-line flow detector (IN/US System, Inc., Tampa, FL) to determine the distribution of radioactivity (Fang et al., 2001; 2004).

Uptake and Metabolism of CUDA and AUDA ---- The COS-7 cells in 75 cm^2 tissue culture flasks were incubated with either 10 μM CUDA or AUDA for various times. After incubation, the cells were harvested and extracted with a 2:1 (v/v) mixture of chloroform/methanol. The extracts were hydrolyzed for 1 h at 50° C in 0.5 ml of methanol containing 50 μl of 0.2 N NaOH and 10% H_2O, and the reactants were further extracted with ethyl acetate and analyzed by high performance liquid chromatography combined with tandem mass spectrometry (LC/MS-MS). The LC/MS-MS analysis was carried out using a Micromass Quattro Ultima triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) interface. The HPLC system consisted of a Waters model 2790 separations module (Waters, Milford, MA) equipped with a Waters model 2487 dual wavelength absorbance detector. The mass spectrometer was coupled to the outlet of the HPLC column (XTerra MS C_{18} column, 30×2.1 mm i.d., 3.5 μm). Ten microliters of samples were injected onto the column, and the separation was done with Solvents A and B containing 0.1 % formic acid and acetonitrile containing 0.1 % formic acid, respectively. The mobile phases were mixed with a linear gradient

from 40 % B to 100 % B over 0 to 5 min, and then held for 8 min with 100 % B at a flow rate of 0.3 mL/min. The ESI was performed in the positive mode. Identification of sEH inhibitor metabolites was carried out with full scan and/or daughter ion scan in the positive and negative mode. Data were acquired in the multichannel analysis mode and continuum mode, and quantitative analysis was performed in the multiple reaction monitoring mode. The data were processed with MassLynx software (Version 3.5) (Watanabe et al., 2001).

sEH Activity Measurement ---- The effect of CUDA and 2-(4-(2-(1-Cyclohexanebutyl-3-cyclohexylureido)ethyl)phenylthio)-2-methylpropionic acid (GW7647), an ureidothioisobutyric acid derivative that has improved lipid-lowering activity compared to fenofibrate (Brown et al., 2001), on recombinant murine sEH activity was determined as described previously (Widstrom et al., 2003). Briefly, a working stock of 13 nM sEH in assay buffer containing 0.75 µM BSA was prepared. [^{3}H]14,15-EET (0.6 µM) was added to 155 µL of assay buffer containing 5 nM BSA in glass vials. Reactions were initiated by adding sEH to a final concentration of 0.4 nM and incubated for 5 min at 30ºC. Reaction mixtures that included CUDA, GW7647 or DMSO (control) were incubated for 2 min prior to enzyme addition. The reactions were terminated by transfer into 5 mL of chloroform/methanol (2:1), followed by addition of 1 mL of 0.9% saline. Tubes were vortexed and centrifuged for 10 min at 4ºC. After the bottom layer was removed and saved, the top layer was re-extracted with 1 mL of chloroform/methanol/0.9% saline (86:14:1). The bottom phases were combined, dried under nitrogen, resuspended in chloroform/methanol (2:1), and applied to silica gel G thin-layer chromatography (TLC) plates and developed to 4 cm in chloroform/methanol/acetic acid (60:30:1). The plate was dried and then further developed in hexane/ethyl acetate/acetic acid (70:30:1). Analysis of the TLC plates with a radioisotope

scanner (Bioscan, Washington, DC) demonstrated the presence of two radioactive peaks that were identified as EET and DHET by comigration with standards.

Statistical Analysis ---- All data are expressed as mean ± SD. Differences between mean values of two groups were analyzed by Student's *t* tests. Differences between mean values of multiple groups were analyzed by one-way analysis of variance with a Newman-Keul post hoc analysis. Probability values of 0.05 or less were considered to be statistically significant.

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RESULTS

PPARα Expression and Activation by different urea-derived selective sEH inhibitors in COS-7 Cells ---- We first established a reliable assay system for PPARα activity. The PPARα protein was not detected in COS-7 cells under control conditions, whereas it was readily detected when the COS-7 cells were transfected with the plasmid containing the mouse PPARα gene. After 18 h of incubation, Wy-14643 (20 μM), a fibric acid derivative that is widely used as a PPARα activator (Devchand et al., 1996), did not increase the PPARα activity in control conditions, but caused a 10-fold increase in PPARα activity in cells that overexpressed PPARα. We next determined the ability of different urea-derived selective sEH inhibitors to activate PPARα in the transfected COS-7 cells (**Figure 1, upper left**). Incubation of the cells with 10 μM CUDA for 18 h increased PPARα activity 5-fold as measured by the luciferase assay. By contrast, no increase in luciferase activity was produced by either10 μM DCU or 10 μM CDU, which contains the dodecyl hydrocarbon chain but no terminal carboxyl group.

A time-dependence study with 10 μM CUDA indicated that a 2-fold increase in luciferase activity was produced after 3 h of incubation, the earliest time point tested (**Figure 1, upper right**). Luciferase activity gradually increased as the incubation continued, and a 4-fold increase was observed at 18 h, the longest time tested. A similar time-dependent response was observed with 20 μM Wy-14643, except that the luciferase activity increased 4-fold after 3 h and reached 10-fold at the end of the 18 h incubation. By contrast, no increase in luciferase activity occurred when the transfected cells were incubated in a control medium that did not contain a PPARα agonist.

Concentration-dependence studies indicated that CUDA produced an increase in luciferase activity in the transfected cells at a threshold concentration of 3 μM (**Figure 1, lower left**). A 6-fold increase in luciferase activity occurred when the cells were incubated for 18 h with 10 μM CUDA, the highest concentration tested. A 3-fold increase in luciferase activity also

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occurred when the transfected cells were incubated for 18 h with 10 μM AUDA (**Figure 1, lower right**), which like CUDA, contains a dodecyl chain with a terminal carboxyl group.

PPARδ and PPARγ Activity ---- To determine whether the stimulatory effect of CUDA was selective for PPARα, additional studies were done with COS-7 cells transfected with plasmids containing the mouse PPARδ or PPARγ genes. No increase in luciferase activity was produced by incubation with 10 μM CUDA in the cells that expressed PPARγ, whereas 10 μM ciglitizone, a selective PPARγ agonist, increased luciferase activity 5-fold. Additional studies indicated that 10 μM CUDA also did not increase luciferase activity in COS-7 cells that expressed PPARδ. Likewise, incubation with 10 μM AUDA did not increase luciferase activity driven by PPARγ or PPARδ in the transduced COS-7 cultures.

Effect of sEH Inhibitors on 14,15-EET Metabolism ---- To determine whether the difference in the ability of CUDA and AUDA as compared with CDU to activate PPARα-dependent gene expression might be due to differences in their effects on EET metabolism, we investigated the effects of these inhibitors on [^{3}H]14,15-EET metabolism by the COS-7 cells. When the cultures were incubated for 1 h with 1 μM [^{3}H]14,15-EET, 48% of total radioactivity was incorporated into the cell lipids. As shown in **Figure 2**, HPLC analysis of the culture medium demonstrated that [^{3}H]14,15-DHET was the major metabolite formed by cultures incubated in a control medium. By contrast, the formation of [^{3}H]14,15-DHET was reduced substantially when the cultures were incubated in media containing 10 μM CDU, CUDA or AUDA. As shown in **Table 1**, the reductions produced by these inhibitors were between 90 and 95%, and the small differences were not statistically significant. These sEH inhibitors did not affect the uptake of

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[^{3}H]14,15-EET by the cells, and transfection with the PPARα gene did not affect [^{3}H]14,15-EET metabolism. The HPLC analysis of the medium demonstrated that in addition to 14,15-DHET, radiolabeled metabolites with retention times of 33, 27 and 11 min accumulated when 10 μM CDU, CUDA or AUDA were added (**Figure 2**). Only very small amounts of these radiolabeled metabolites were detected in the medium of control cultures. The metabolites with retention times of 33 and 27 min have been identified previously as the 14,15-EET β-oxidation products 10,11-epoxyhexadecadienoic acid (10,11-epoxy-16:2) and 8,9-epoxytetradecaenoic acid (8,9-epoxy-14:1), respectively (Fang et al., 2000a). Consistent with these findings, previous studies with porcine coronary endothelial cultures demonstrated that inhibition of sEH by DCU also increased the conversion of [^{3}H]14,15-EET to 10,11-epoxy-16:2 and 8,9-epoxy-14:1 (Fang et al., 2001). We have not been able to positively identify the metabolite with a retention time of 11 min.

Effect of CUDA on PPARα protein expression and competitive binding of CUDA to the ligand binding domain of PPARα ---- To determine whether CUDA altered expression of the PPARα protein, the control cells or cells transfected with the PPARα gene were incubated with 10 μM CUDA or 20 μM Wy-14643 for 18 h. CUDA did not induce PPARα protein in the control cells or cause an increase in the amount of PPARα protein expressed by the transfected cells. A similar result was obtained with Wy-14643 (**Figure 3, top panel**). To determine whether CUDA can bind to the ligand binding domain of PPARα, the mPPARα-LBD-GST proteins were incubated with 300 nM [^{3}H]Wy-14643 and 5 or 25 μM CUDA or 5 μM non-labeled Wy-14643. The binding of [^{3}H]Wy-14643 to the ligand binding domain of mPPARα-LBD was competitively displaced by Wy-14643 or CUDA (**Figure 3, bottom panel**). A 56% reduction in

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[^{3}H]Wy-14643 binding was produced by 5 μM non-labeled Wy-14643. 5 μM CUDA decreased [^{3}H]Wy-14643 binding by 28%, and 25 μM CUDA decreased the binding by 46%.

Metabolism of CUDA and AUDA by COS-7 Cells ---- We investigated the metabolism of CUDA and AUDA to determine whether they are converted to products that may have an effect on their ability to activate PPARα. After incubation of the COS-7 cells with either 10 μM CUDA or AUDA for 6 h, products contained in the cells and medium were analyzed by LC/MS-MS. **Figure 4** illustrates the tandem mass chromatograms obtained and the structures of these compounds. Optimization of tandem MS conditions allowed for complete separation of the metabolites. To identify them, the medium extracts were analyzed with full scan and daughter ion scan in the positive and negative mode. Due to lack of standards, the amounts of the metabolites contained in the cells and medium were estimated from the calibration curves for the parent compounds.

Figure 5 shows the distribution of these compounds in the cells and medium at the end of the 6 h incubation. The media contained 90- to 170-times more of these compounds than the cells. β-Oxidation products accounted for almost all of the material present in the media, whereas about half of the material contained in the COS-7 cells was either unmodified CUDA or AUDA. The most abundant CUDA metabolite recovered from the medium contained a 10-carbon fatty acid chain, whereas the most abundant AUDA metabolite contained an 8-carbon fatty acid chain. The main AUDA β-oxidation product present in the cells also contained an 8-carbon fatty acid chain, whereas metabolites with 10- and 6-carbon fatty acid chains were the main CUDA derivatives recovered in the cells. These findings indicate that both CUDA and AUDA are substrates for partial β-oxidation in COS-7 cells and that while most of the β-oxidation products

are released and accumulate in the medium, small amounts of these chain-shortened products are retained in the cells.

Effect of CUDA metabolites on PPARα activity and binding to the ligand-binding domain of PPARα ---- Additional studies were done **t**o determine whether CUDA metabolism affects its ability to activate PPARα. The COS-7 cells were incubated with 10 μM CUDA and after 18 h, the medium was collected and transferred to other transfected COS-7 cells that has not been exposed to CUDA. The incubation was then continued for an additional 18 h. Activation of PPARα by the post-incubation medium was decreased by 50% as compared with medium containing CUDA [6.34 ± 1.2 (10 μM CUDA) *vs* 3.16 ± 0.15 (medium from cells that had been incubated with 10 μM CUDA), P<0.01]. These results suggested that CUDA metabolites have less capacity to activate PPARα. This observation was confirmed by incubating synthetic CUDA metabolites with the transfected cells. *N*-cyclohexyl-*N'*-octanoic acid urea (CUOA), a metabolic product of CUDA formed following two cycles of ß-oxidation, caused only a 3-fold increase in PPARα activity, and *N*-cyclohexyl-*N'*-hexanoic acid urea (CUHA), a metabolic product of CUDA formed following three cycles of ß-oxidation, did not increase the PPARα activity as compared with the control (**Figure 6. top**). Competitive binding analyses indicated that the chain-shortened metabolite CUOA displaced less [^{3}H]Wy-14643 from the ligand binding domain of mPPARα-LBD as compared with CUDA, a 20% decrease as compared to a 50% decrease produced by CUDA. Moreover, CUHA did not reduce the binding of [^{3}H]Wy-14643 to PPARα (**Figure 6, bottom**).

Effect of CUDA on expression of the PPARα response gene CPT1A in HepG2 cells ---- We determined whether activation of PPARα by CUDA will up-regulate a PPARα responsive gene.

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Because PPARα responsive genes have been identified in human HepG2 cells (Hsu et al., 2001), this cell line was used for these studies. CUDA (10 μM) caused a 2-fold increase of PPARα activity as measured by luciferase activity in transfected HepG2 cells, and Wy-14643 (10 μM) increased the PPARα activity 5-fold (**Figure 7, top**). A real-time-PCR assay indicated that CUDA, as well as Wy-14643, significantly increased the level of CPT1A mRNA, a PPARα responsive gene, in transfected HepG2 cells (**Figure 7, bottom**).

Effect of PPARα agonists on sEH activity ---- To determine whether compounds widely used as PPARα agonists affect sEH activity, we investigated the effects of Wy-14643 and GW7647 on [^{3}H]14,15-EET metabolism by the COS-7 cells. As shown in Figure 8, 10 μM Wy-14643 or 0.1 μM GW7647, concentrations that are commonly used to produce PPARα activation, did not reduce the conversion of [^{3}H]14,15-EET to DHET (**Figure 8, left**). However, the formation of [^{3}H]14,15-DHET was reduced by 50% when the cultures were incubated in media containing 10 μM GW7647. HPLC analysis of the medium following incubation of the COS-7 cultures with [^{3}H]14,15-EET indicated that in addition to 14,15-DHET, 10,11-epoxy-16:2 and 8,9-epoxy-14:1 accumulated when 10 μM GW7647 was added, a pattern similar to that seen with other sEH inhibitors (**Figure 8, right**). The inhibitory effect of high concentration of GW7647 was further demonstrated in studies with recombinant murine sEH (**Figure 9**). When the sEH was incubated for 5 min with 0.6 μM [^{3}H]14,15-EET, 60% of the total radioactivity was converted to [^{3}H]14,15-DHET. The formation of [^{3}H]14,15-DHET was completely inhibited by 1 μM CUDA and reduced by 30% by 1 μM GW7647.

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DISCUSSION

We have observed that CUDA and AUDA, two potent urea-derived sEH inhibitors that contain a carboxylic acid substitution, are activators of PPARα. The effects are specific for PPARα, because neither compound activated PPARδ or PPARγ, and the activation of PPARα by CUDA and AUDA appears to be independent of sEH inhibition. The dual function of these compounds, inhibition of sEH and activation of PPARα, suggests a unique pharmacological profile that may be of considerable value in treating cardiovascular disease.

CDU, which differs from CUDA only by the absence of a carboxyl group at the end of the *N'*-dodecyl chain, did not activate PPARα. This suggests that the carboxylic acid group present in CUDA and AUDA plays a key structural role in the activation process, a finding consistent with the previous observation that long-chain fatty acids activate PPARα but the corresponding fatty alcohols do not (Forman et al., 1997). The fact that CUDA is more efficacious than AUDA in activating PPARα in the transduced COS-7 cells suggests that the *N*-cyclohexyl group facilitates the interaction more effectively than the corresponding *N*-adamantanyl group. In this regard, CUDA is structurally similar to GW7647, a potent PPARα agonist that also is a urea derivative containing a *N*-cyclohexyl group (Brown et al., 2001). However, in addition to structural features, differences in the biopharmaceutical properties of these compounds also may account for their relative potency in activating PPARα.

The possibility that CUDA and AUDA do not directly bind to PPARα but instead facilitate the binding of an endogenous ligand was initially considered. For example, the 19- and 20-hydroxylated derivatives of EETs are PPARα ligands (Cowart et al., 2002), suggesting that sEH inhibition might activate PPARα by increasing the intracellular accumulation of EET metabolites. However, CDU inhibited the conversion of 14,15-EET to DHET to about the same

extent as CUDA and AUDA but did not increase PPARα activity, indicating that the activation of PPARα by CUDA and AUDA is independent of their inhibitory effect on the conversion of EET to DHET. Likewise, the increased accumulation of 14,15-EET partial β-oxidation metabolites that occurred when the inhibitors were added appears to be unrelated to PPARα activation because CDU also increased the accumulation of these products.

Our data suggest that CUDA and AUDA most likely activated PPARα through a ligand-dependent mechanism. As was observed with Wy-14643, CUDA did not increase the amount of PPARα protein in the COS-7 cells and only increased PPARα-dependent luciferase activity in those cultures that expressed PPARα. Furthermore, small amounts of unmodified CUDA and AUDA were recovered in the cells in incubations lasting 6 h, indicating that these compounds probably are available intracellularly for an extended period. Many fatty acids and eicosanoids activate PPARα through a binding mechanism (Yu et al., 1995; Willson et al., 1997; Murakami et al., 1999; Bishop-Bailey et al., 2003), and the fact that CUDA and AUDA are fatty acid derivatives suggests that this structural property enables these compounds to also bind to PPARα. Consistent with this notion, CUDA competitively inhibited the binding of Wy-14643 to the ligand binding domain of mPPARα-LBD, indicating that direct binding to PPARα can occur. Activation of PPARα mediates induction of mitochondrial, microsomal, and peroxisomal fatty acid oxidation, and CPT1A gene is a PPARα responsive gene in human HepG2 cells (Hsu et al., 2001). Overexpression of mPPARα in HepG2 cells does not increase the induction of CPT1A, but it causes a significant increase when the cells are treated with Wy-14643 (Hsu et al., 2001). We found that, similar to Wy-14643, CUDA increased the PPARα activity as measured by the luciferase activity, and it caused a 2-fold induction of CPT1A mRNA. These results suggest that CUDA can function as a PPARα agonist in human cells.

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Analysis by tandem mass spectrometry revealed that most of the CUDA and AUDA added to the cultures was converted to β-oxidation products during the first 6 h of incubations with the COS-7 cells. The carboxylic acid chains of these β-oxidation products, which accumulated primarily in the medium, contained 4 to 10 carbons. This β-oxidation process most likely is the catabolic mechanism that inactivates CUDA and AUDA. Indeed, the chain-shortened CUDA metabolites have decreased abilities to activate PPARα and bind weakly to the ligand binding domain of mPPARα-LBD compared to CUDA (**Figure 6**). This is supported by previous studies with sEH inhibitors in which the terminal carboxylic acid is esterified. The inhibition of sEH produced by these esters, which are as potent as the corresponding carboxylic acid compounds, decreases as the chain becomes shorter, and the 4-carbon derivative is essentially inactive (Morriseau et al., 2002).

The concentrations of CUDA and AUDA that produced appreciable activation of PPARα in the COS-7 cells, although in the same range as the effective concentration of Wy-14643, are substantially higher than those needed to inhibit sEH. For example, the IC_{50} values for CUDA and AUDA inhibition of mouse and human recombinant sEH are between 10 and 100 nM (Morisseau et al., 2002). However, CUDA and AUDA were designed for selective sEH inhibition, without regard to PPARα activation. Many structurally related compounds retain a high degree of sEH inhibitory activity (Morisseau et al, 1999; Morisseau et al, 2002), making it quite likely that either CUDA or AUDA could be structurally modified to increase its effectiveness for PPARα activation without a substantial loss of sEH inhibitory activity. For example, because β-oxidation is an inactivation process for CUDA, adding methyl groups to the β-carbon is likely to block this process (Spector et al., 1965), thereby enhancing its intracellular

activity of CUDA as a PPARα activator. Similar methyl-branched structures are present in two PPARα activators, ciprofibric acid and clofibric acid (Forman et al., 1997).

GW7464, which is a very potent PPARα agonist (EC50 = 6 nM for PPARα activation), contains a cyclohexyl urea group. Therefore, we tested whether it also can inhibit sEH activity. Although no sEH inhibition was observed with 100 nM GW7464, a concentration that activates PPARα, GW7464 inhibited sEH activity at high concentrations in cultured COS-7 cells, and when incubated with recombinant sEH. However, the inhibitory effect of GW7464 on sEH was weaker than the inhibition produced by CUDA. These observations further suggest that structural modification of either CUDA or GW7464 are likely to produce novel compounds that combine potent PPARα activation with selective sEH inhibition.

Activation of PPARα decreases cholesterol esterification in macrophages and increases the removal of cholesterol from human macrophage foam cells (Chinetti et al., 2001; Chinetti et al., 2003; Ricote et al, 2004). In addition to these effects on lipids, PPARα activation inhibits vascular smooth muscle activation and has anti-inflammatory effects (Staels et al., 1998; Fruchart et al., 1999; Delerive et al., 1999; Delerive et al., 2000). It also produces many favorable effects on vascular function, such as diminishing oxidative stress, antagonizing the actions of angiotensin II, and reducing blood pressure in rodent models of hypertension (Schiffrin et al., 2003). Therefore, in addition to preserving EETs, activation of PPARα by CUDA or AUDA could have favorable effects on vascular function, the regulation of lipid metabolism, and inflammatory processes that facilitate atherosclerosis (Plutzky, 2003).

Another potential benefit of PPARα activation by CUDA or AUDA, as opposed to activators that do not inhibit sEH activity, is suggested by the fact that PPARα increases sEH expression in rodent species. For example, sEH mRNA levels in mouse liver, heart, and kidneys

are increased by clofibrate, a hypolipidemic drug that activates PPARα (Hammock et al., 1983; Johansson et al., 1995). Because sEH activity is associated with blood pressure elevations in some experimental systems (Sinal et al., 2000; Yu et al., 2000; Imig et al, 2002; Spector et al, 2004), increased sEH expression is a potentially undesirable consequence of PPARα activation. However, any increase in sEH expression produced by CUDA or AUDA should be compensated by their potent inhibitory effect on sEH activity.

Hypertension is a major risk factor for atherosclerotic cardiovascular disease, and selective sEH inhibitors are being tested in animal models of hypertension (Yu et al., 2000). The use of sEH inhibitors like CUDA or AUDA may have the added benefit of PPARα activation. Therefore, further development of this novel class of compounds that has combined effects on sEH and PPARα could represent a useful new pharmacological approach for the prevention and treatment of cardiovascular disease.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Activation of PPARα by sEH inhibitors. The COS-7 cultures were transfected with β-Gal, PPARα and the luciferase reporter genes in a 24 h incubation. The cultures then were incubated for 18 h with 1 mL control medium or media containing 10 μM DCU, CDU or CUDA, and the luciferase and β-Gal activities in cell lysates were measured. The luciferase activity was normalized to the ß-Gal activity. The results are expressed as fold-activation relative to the vehicle control (**upper left).** In the time- dependent study (**upper right**), the cultures were incubated with 1 mL control medium, 10 μM CUDA, or 20 μM Wy-14643. In the CUDA concentration study (**lower left)** and AUDA concentration study (**lower right**), the cultures were incubated with these compounds for 18 h. The data are present as mean ± SD values obtained from at least three separate experiments. * $P<0.05$, ** $P<0.01$ *vs*. control.

Figure 2. Effect of sEH inhibitors on 14,15-EET metabolism. COS-7 cells were incubated initially in 1 ml DMEM medium containing 0.1 μM albumin at 37° C, with or without 10 μM sEH inhibitors. After 30 min, 1 μM [^{3}H]14,15-EET was added and the incubations continued for 1 h. The media were collected and extracted, the lipid extracts separated by reverse-phase HPLC, and the radiolabeled compounds detected with an in-line flow scintillation counter. Radiochromatograms from single cultures are shown, but similar results were obtained from two additional cultures in each case.

Figure 3. Effect of CUDA on expression of PPARα protein, and binding of CUDA to PPARα protein. COS-7 cultures (~60% confluent) were incubated for 24 h at 37°C with or without 0.02 μg of the pCMX-mPPARα expression vector. The cultures were then incubated for

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an additional 18 h with 1 mL control medium containing vehicle alone, 10 μM CUDA or 20 μM Wy-14643. The PPARα and β-actin proteins in the cell lysates were detected by western blotting (**top panel**). For binding assays, 2 μg GST-PPARα-LBD fusion protein was incubated with 300 nM [^{3}H]Wy-14643 in a total volume of 50 μl in the presence of ethanol, unlabeled Wy-14643, or various concentrations of CUDA. The free and bound ligands were separated on a Sephadex G-25 column, and the amount of bound [^{3}H]Wy-14643 was then determined by liquid scintillation counting (**bottom panel**). The binding assays were repeated in quadruple sets and presented as mean ± S.D. ** P<0.01 *vs*. controls.

Figure 4. Identification of CUDA and AUDA metabolites. COS-7 cells in 75-cm^2 flasks were incubated with 5 mL medium containing either 10 μM CUDA or AUDA. After incubation, the media were collected, extracted with ethyl acetate, and assayed for metabolites by LC/MS-MS. In order to obtain sufficient quantities of products for structural identification, samples from two separate cultures subjected to identical incubation conditions were combined. The tandem mass chromatograms and the structures of compounds contained in the medium after incubation for 6 h are shown. The "n" value indicates the number of carbon atoms contained in the fatty acid chain of the CUDA and AUDA metabolites.

Figure 5. Distribution of CUDA and AUDA and their products in COS-7 cells and medium. The incubation conditions and methods of analysis were the same as described in Figure 4.

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Figure 6. Activation and the binding of PPARα by CUDA metabolites. The COS-7 cultures were transfected with β-Gal, PPARα and the luciferase reporter genes in a 24 h incubation. The cultures then were incubated for 18 h with 1 mL control medium, or media containing either 10 μM CUDA, or the chain-shortened CUDA metabolites. The luciferase and β-Gal activities in the cell lysates were measured, and the results are expressed as fold-activation relative to the vehicle control (mean ± SD, n=4). ** P<0.01 *vs*. control. For binding assays, 2 μg GST-PPARα-LBD fusion protein was incubated with 300 nM [^{3}H]Wy-14643 in a total volume of 50 μl in the presence of ethanol, 25 μM CUDA or CUDA metabolites. The free and bound ligands were separated on a Sephadex G-25 column, and the amount of bound [^{3}H]Wy-14643 was determined by liquid scintillation counting. The binding assays were repeated in quadruplicate sets and presented as mean ± S.D. * P<0.05, ** P<0.01 *vs*. controls.

Figure 7. Activation of PPARα by CUDA and effect on CPT1A in HepG2 cells. HepG2 cultures were transfected with β-Gal, PPARα and the luciferase reporter genes in a 24 h incubation. The cultures then were incubated for 18 h with 1 mL control medium or media containing 10 μM CUDA or Wy-14643, and the luciferase and β-Gal activities in cell lysates were measured. The luciferase activity was normalized to the ß-Gal activity. The results are expressed as fold-activation relative to the vehicle control (**top panel).** In a separate experiment under the same conditions, the RNA was extracted from the HepG2 cells and CPT1A mRNA was assayed by real-time RT-PCR. The data are expressed as fold differences from the control cells and have been normalized to the expression of GAPDH (**bottom panel**).

Figure 8. **Effect of PPARα agonists on cellular sEH activity.** COS-7 cells were incubated initially in 1 ml DMEM medium containing 0.1 μM albumin at 37° C, with or without various concentration of PPARα agonists. After 30 min, 1 μM [^{3}H]14,15-EET was added and the incubations continued for 1 h. The media were then collected and extracted, the lipid extracts separated by reverse-phase HPLC, and the radiolabeled compounds detected with an in-line flow scintillation counter. The values are results obtained from 3 separate cultures, expressed as mean ± SD. The specific activity of the added [^{3}H]14,15-EET was used to calculate the pmol amounts (**left panel**). ** P<0.01 *vs.* control. Representative HPLC radiochromatograms from single cultures are shown on **right panel**, but similar results were obtained from two additional cultures in each case.

Figure 9. **Inhibition of recombinant mouse sEH by CUDA and GW7647**. [^{3}H]14,15-EET (0.6 μM) was incubated with vehicle, CUDA or GW7647 for 2 min prior to addition of recombinant mouse sEH. After a 5 min incubation, the reaction was terminated and the lipids were extracted and analyzed by TLC. Representative radioactivity scans of TLC plates are shown. (A) [^{3}H]14,15-DHET production in the absence of inhibitors. (B) [^{3}H]14,15-DHET production in the presence of 1 μM CUDA. (C) [^{3}H]14,15-DHET production in the presence of 1 μM GW7647.

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Table 1. Effect of sEH inhibitors on conversion of [^{3}H]14,15-EET to 14,15-DHET by COS-7 cells

Treatment	[^{3}H]14,15-DHET Formation (pmol/min/mg protein)	Inhibition (%)
Control	26.0 ± 1.1	---
CDU	2.40 ± 0.13**	90.6
CUDA	1.60 ± 0.13**	93.7
AUDA	1.47 ± 0.27**	94.6

The COS-7 cells were incubated initially for 30 min in 1 mL DMEM medium, with or without 10 μM sEH inhibitors. After addition of 1 μM [^{3}H]14,15-EET to the medium, the incubation was continued for 1 h, and the radiolabeled 14,15-DHET contained in the medium was assayed by reverse-phase HPLC as described in Figure 2. The values are results obtained from 3 separate cultures, expressed as mean ± SD. The specific activity of the added [^{3}H]14,15-EET was used to calculate the pmol amounts. ** $P<0.01$ *vs.* control.

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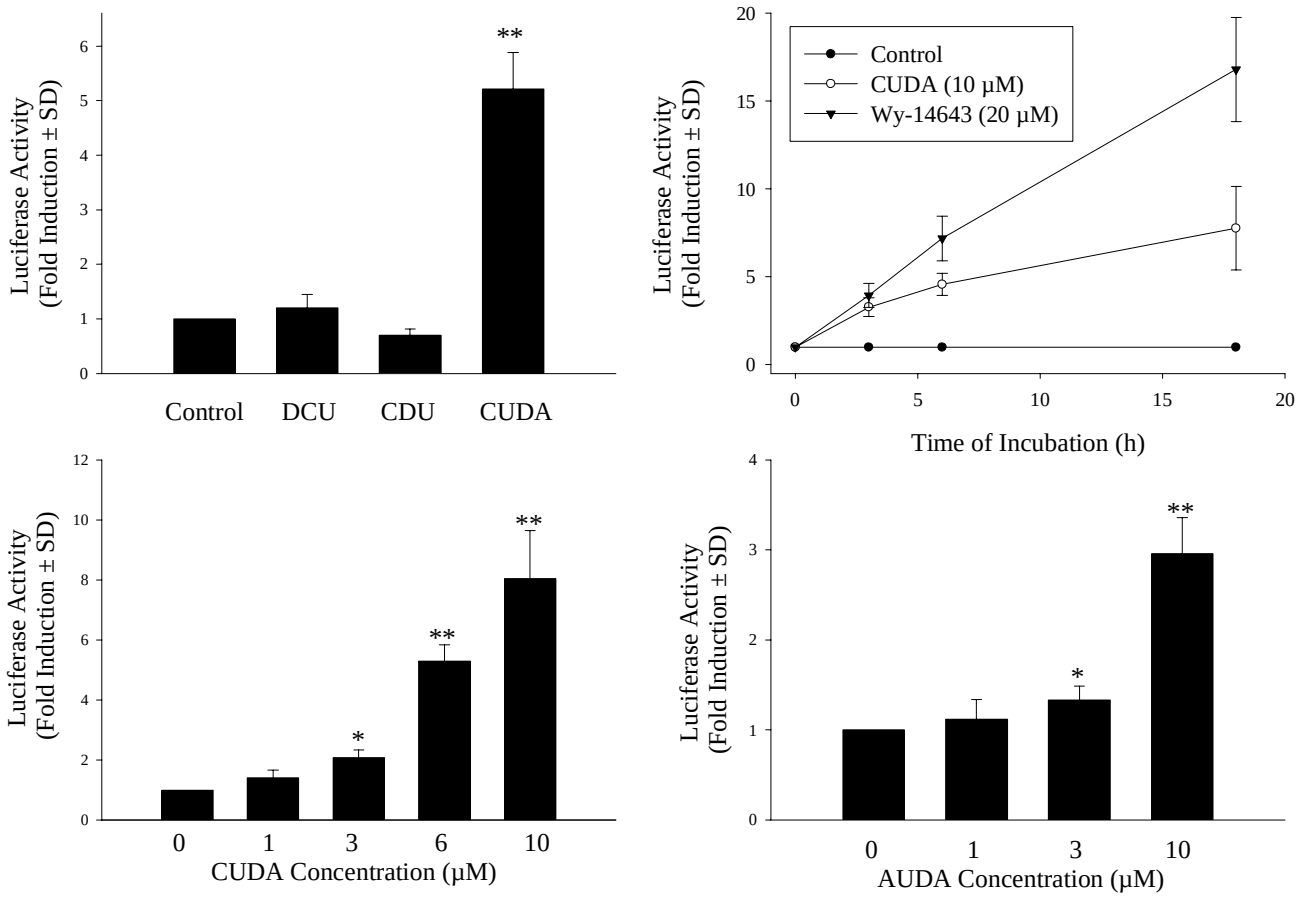

Figure 1

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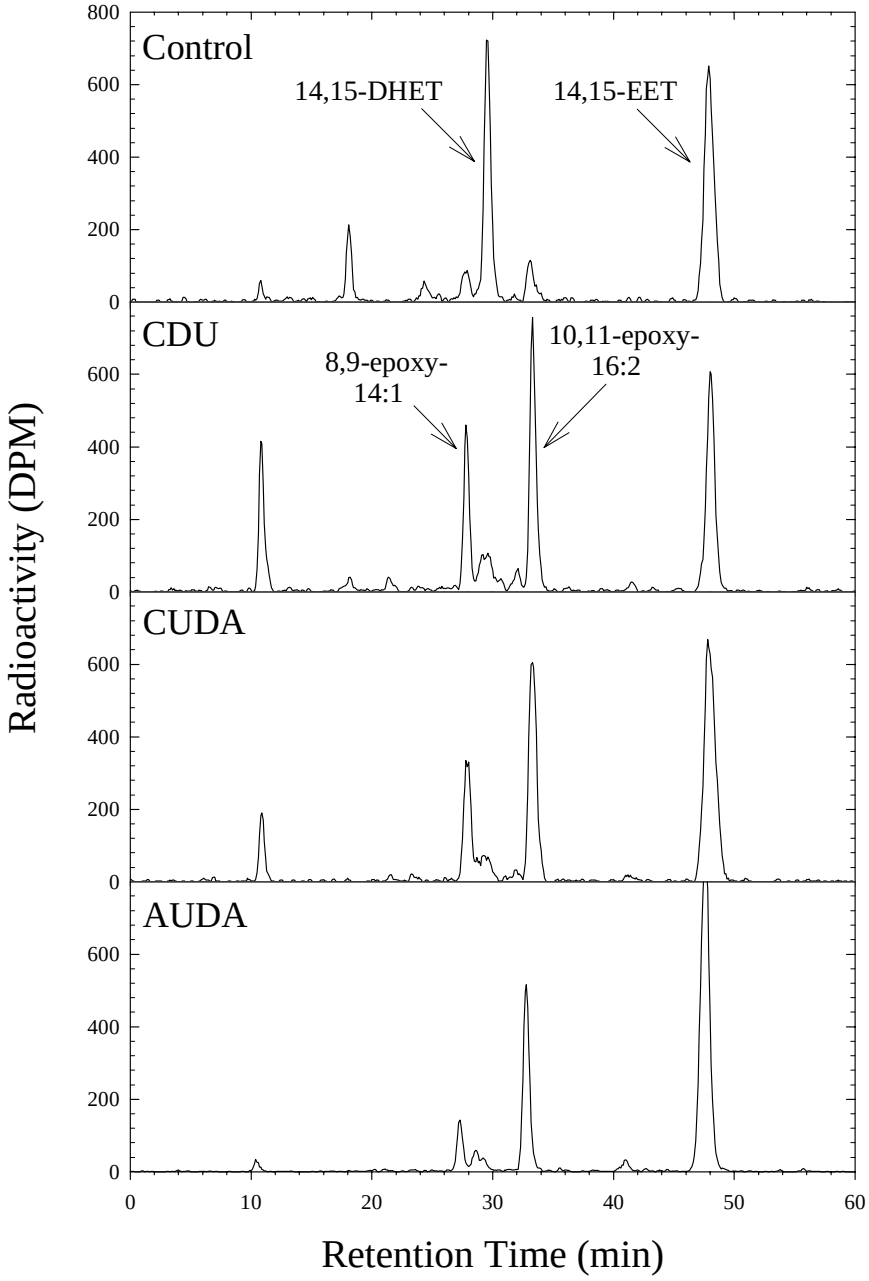

Figure 2

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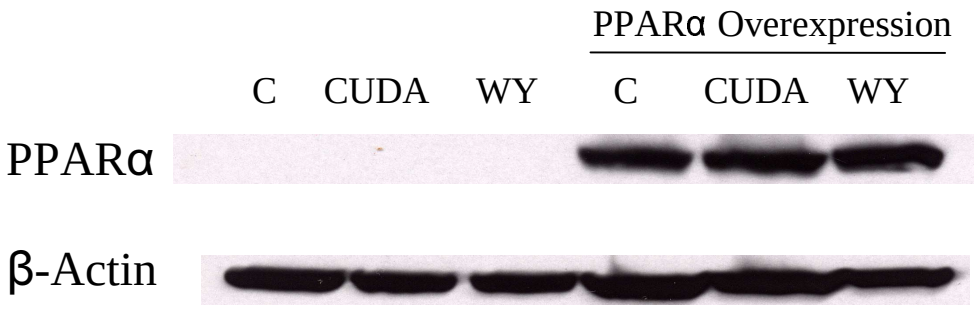

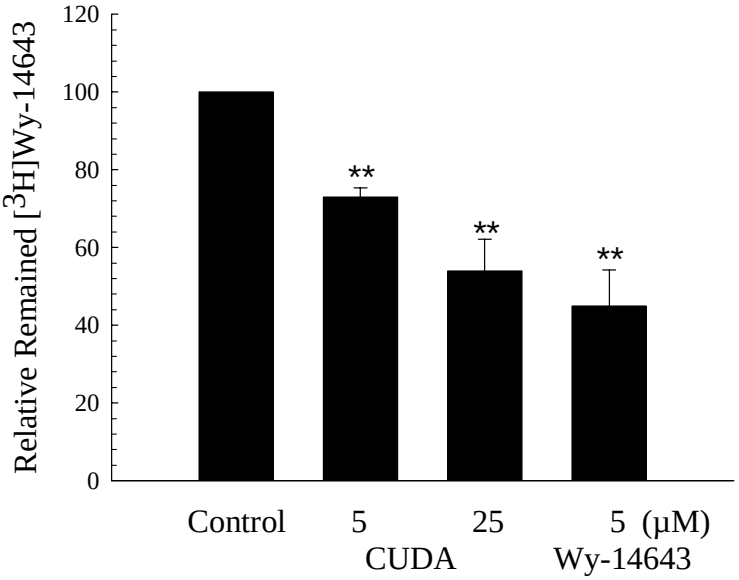

Figure 3

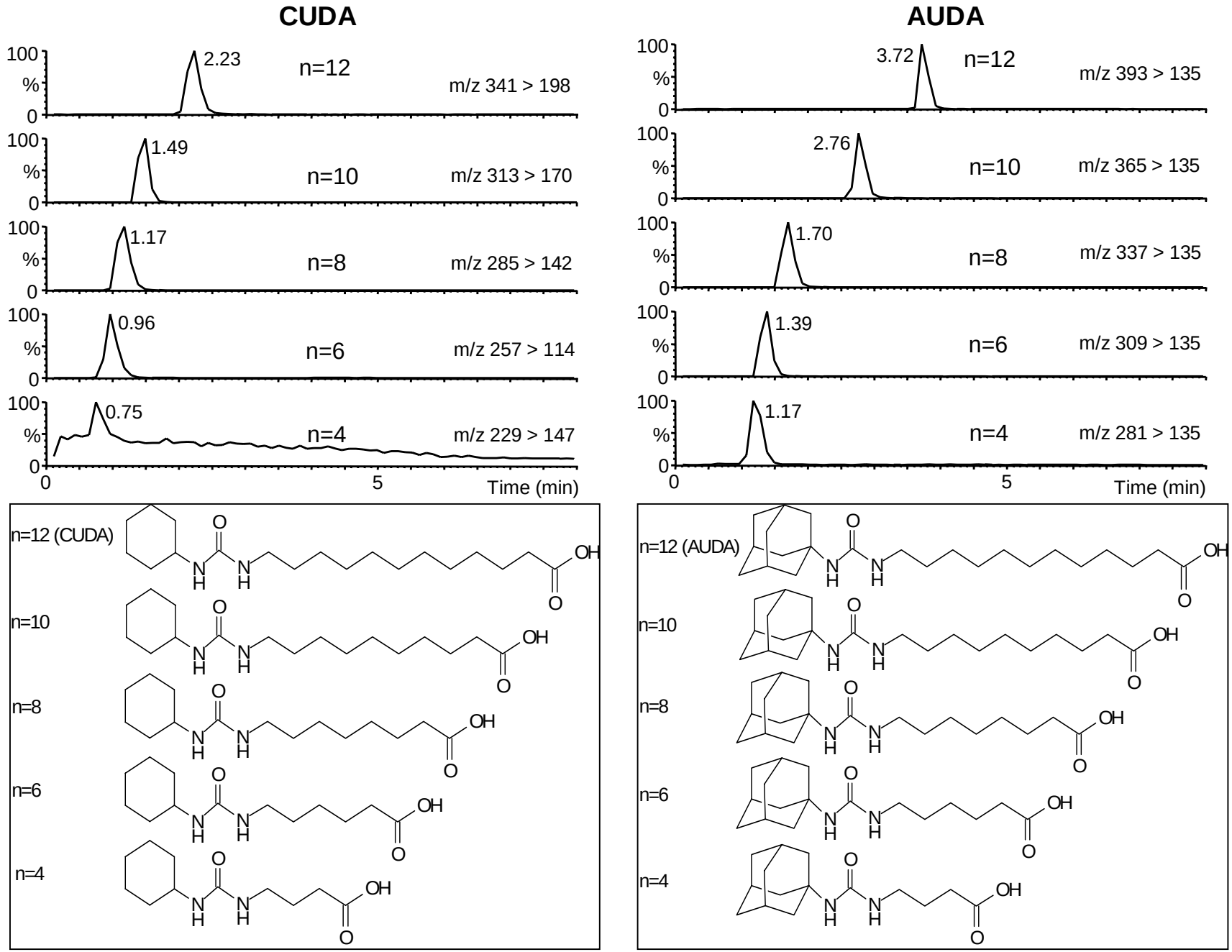

Figure 4

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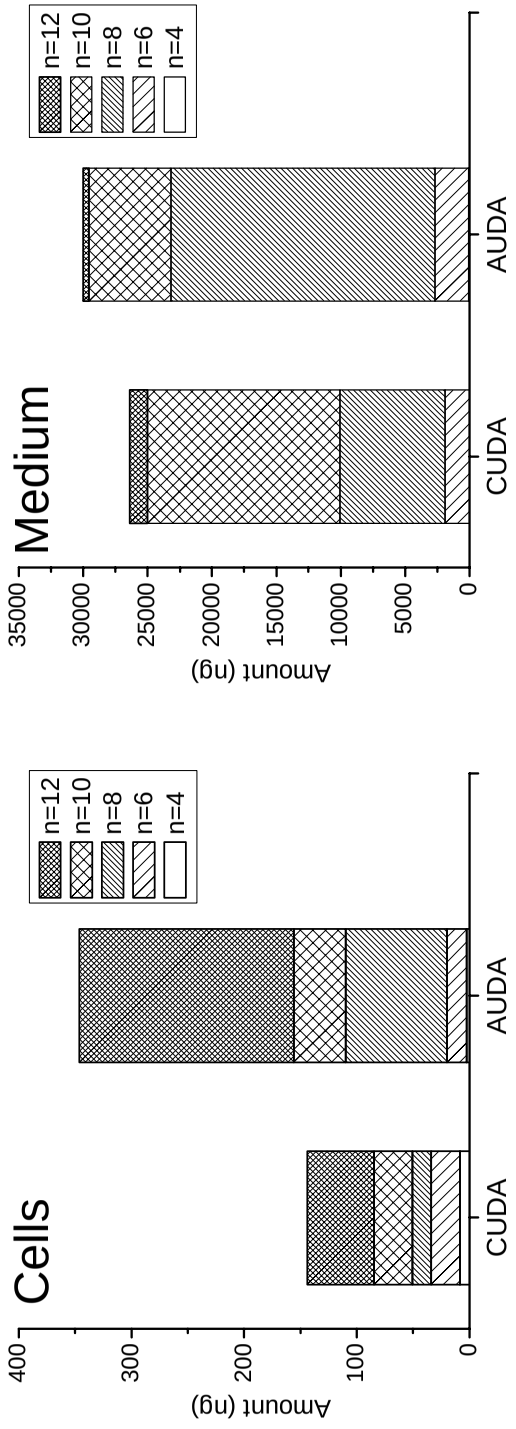

Figure 5

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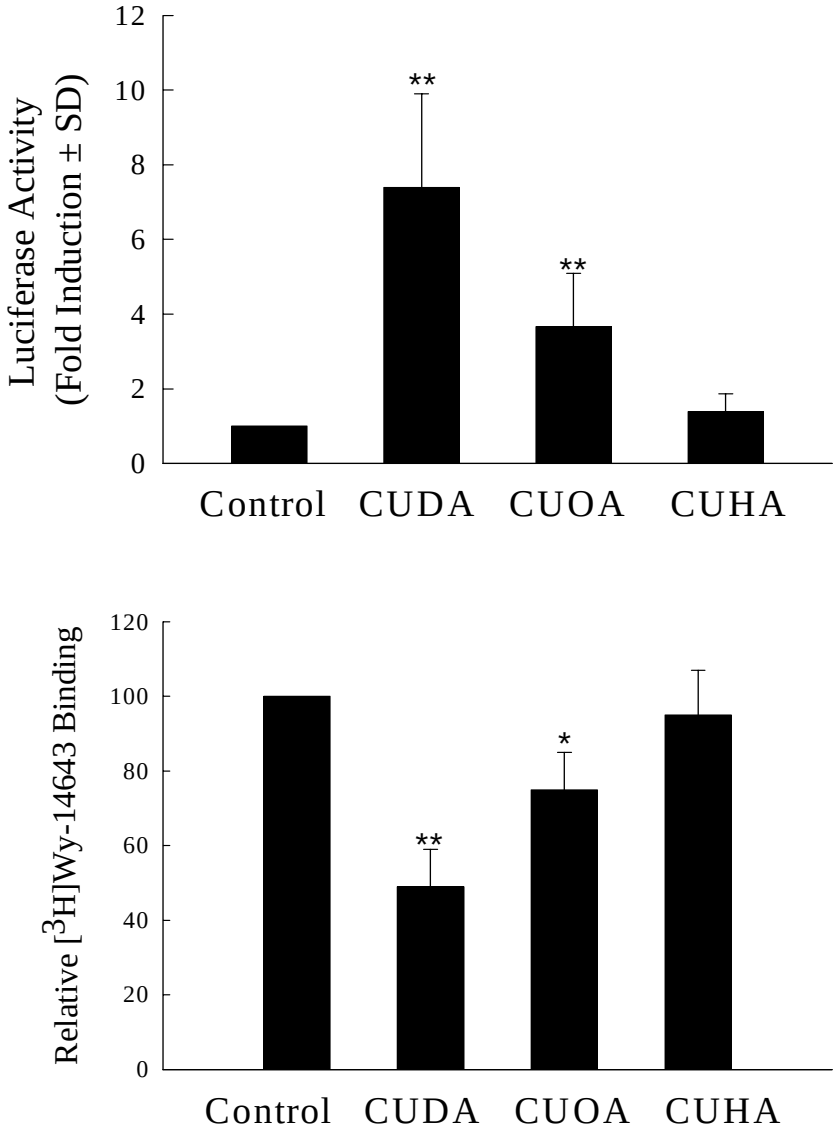

Figure 6

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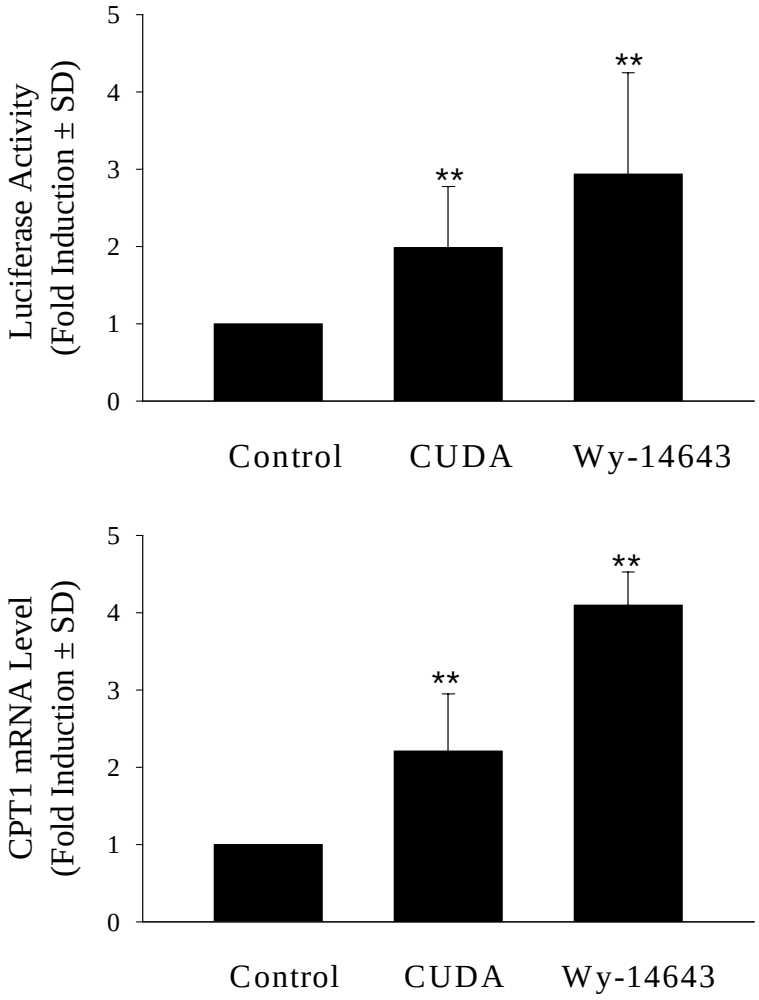

Figure 7

JPET Fast Forward. Published on March 29, 2005 as DOI: 10.1124/jpet.105.085605
This article has not been copyedited and formatted. The final version may differ from this version.

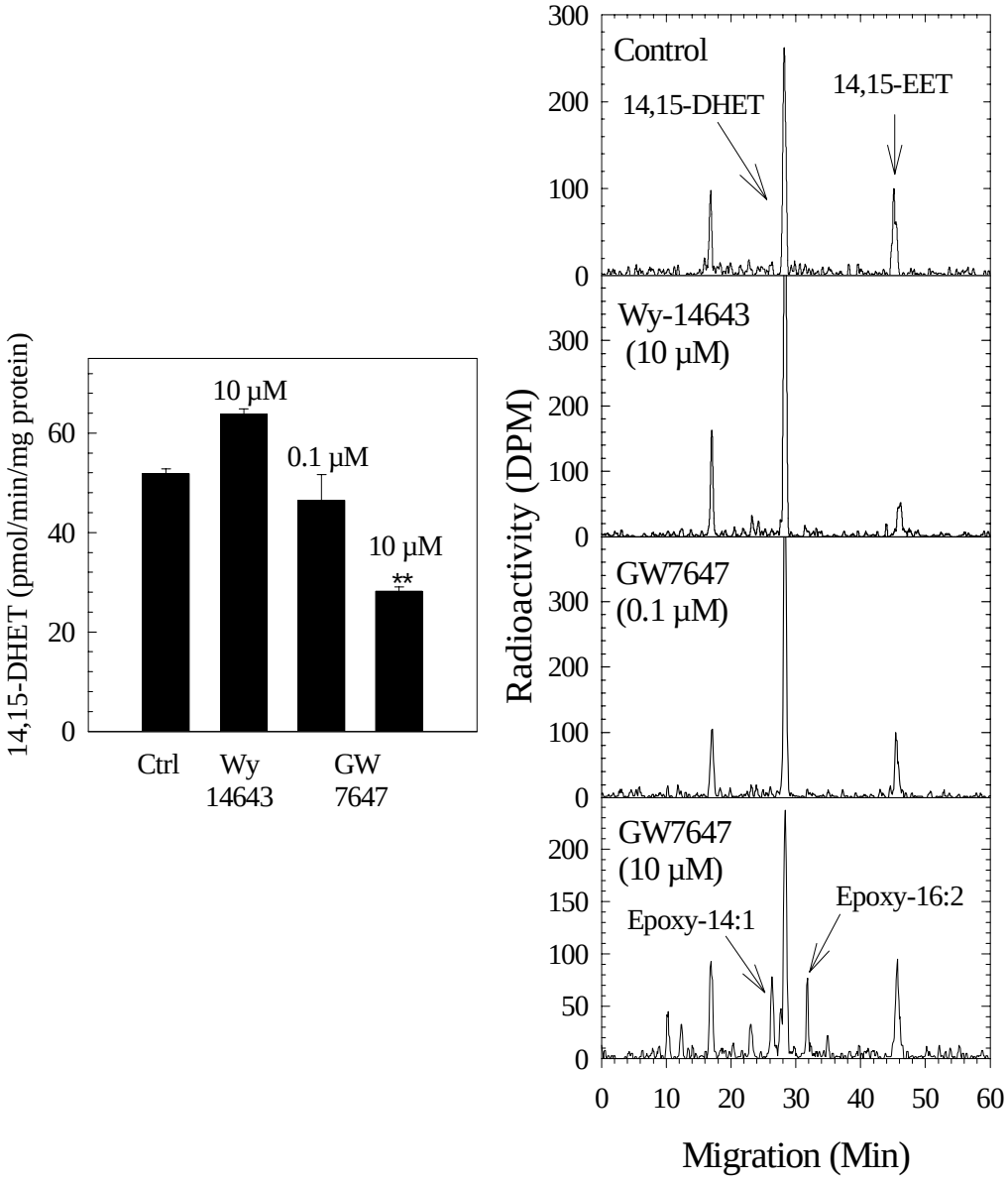

Figure 8

JPET Fast Forward. Published on March 29, 2005 as DOI: 10.1124/jpet.105.085605
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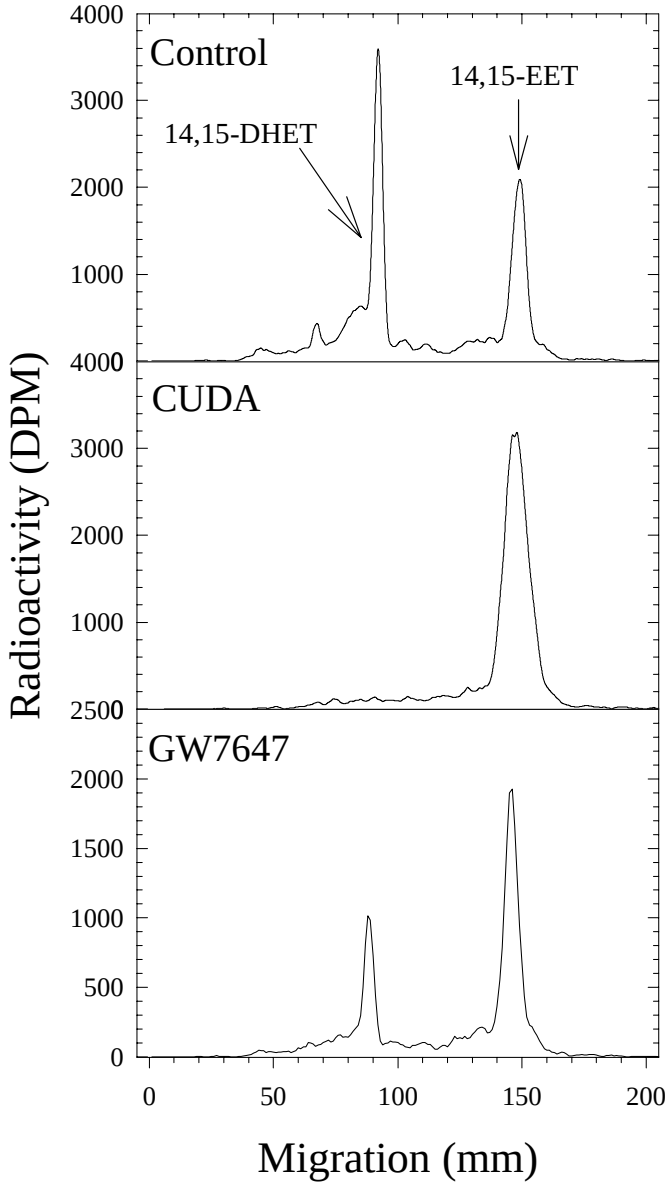

Figure 9