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THE ANTICANCER DRUG ELLIPTICINE IS A POTENT INDUCER OF RAT CYTOCHROMES P450 1A1 AND 1A2, THEREBY MODULATING ITS OWN METABOLISM

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Running title: Ellipticine induce CYP1A1/2

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ABBREVIATIONS: AhR, aryl hydroxarbon receptor; α-NF, α-naphthoflavone; ARNT, AhR nuclear translocator; β-NF, β-naphthoflavone; COX, cyclooxygenase; c_T, cycle threshold; EROD, 7-ethoxyresorufin *O*-deethylation; MDR, multidrug resistance; MROD, 7-methoxyresorufin *O*-deethylation; PVDF, polyvinylidene difluoride; CYP, cytochrome P450; SDS, sodium dodecyl sulfate; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; RAL, relative adduct labelling; RT, real-time; PCR, polymerase chain reaction.

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ABSTRACT:

Ellipticine is an antineoplastic agent, whose mode of action is based mainly on DNA intercalation, inhibition of topoisomerase II and formation of covalent DNA adducts mediated by cytochromes P450 (CYP) and peroxidases. Here, this drug was found to induce CYP1A1 and/or 1A2 enzymes and their enzymatic activities in livers, lungs and kidneys of rats treated (i.p.) with ellipticine. The induction is transient. In the absence of repeated administration of ellipticine, the levels and activities of the induced CYP1A decreased almost to the basal level two weeks after treatment. The ellipticine-mediated CYP1A induction increases the DNA adduct formation by the compound. When microsomal fractions from livers, kidneys and lungs of rats treated with ellipticine were incubated with ellipticine, DNA adduct formation, measured by ^{32}P-postlabelling analysis, was up to 3.8-fold higher in incubations with microsomes from pretreated rats than with controls. The observed stimulation of DNA adduct formation by ellipticine was attributed to induction of CYP1A1 and/or 1A2-mediated increase in ellipticine oxidative activation to 13-hydroxy- and 12-hydroxyellipticine, the metabolites generating two major DNA adducts in human and rat livers. In addition to these metabolites, increased formation of the excretion products 9-hydroxy- and 7-hydroxyellipticine was also observed in microsomes of rats treated with ellipticine. Taken together, these results demonstrate for the first time that by inducing CYP1A1/2, ellipticine increases its own metabolism leading both to an activation of this drug to reactive species forming DNA adducts and to detoxication metabolites, thereby modulating to some extent its pharmacological and/or genotoxic potential.

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Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. 1), an alkaloid isolated from *Apocyanaceae* plants, exhibits significant antitumor and anti-HIV activities (Auclair, 1987; Stiborová et al., 2001). The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity (Auclair, 1987). Nevertheless, ellipticine is a potent mutagen (for a summary, see Stiborová et al., 2001).

Ellipticines are anticancer drugs, whose precise mechanisms of action have not been explained yet. It was suggested that the prevalent mechanisms of their antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA (Auclair, 1987; Singh et al., 1994) and (ii) inhibition of DNA topoisomerase II activity (Auclair, 1987; Fossé et al, 1992; Froelich-Ammon et al., 1995). Ellipticine and its metabolite 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines (Sugikawa et al., 1999) and this correlates with their cytotoxic activity. Ellipticines also uncouple mitochondrial oxidative phosphorylation (Schwaller et al., 1995) and thereby disrupt the energy balance of cells.

We demonstrated that ellipticine covalently binds to DNA after being enzymatically activated with cytochromes P450 (CYP) or peroxidases (Stiborová et al., 2001; 2003c; 2004; 2007; Frei et al., 2002). Human and rat CYPs of 1A and 3A subfamilies are the predominant enzymes catalyzing oxidation of ellipticine either to metabolites that are excreted from organisms (7-hydroxy- and 9-hydroxyellipticine) or form DNA adducts (12-hydroxy- and 13-hydroxyellipticine) (Stiborová et al., 2001; 2003c; 2004; Kotrbová et al., 2006). Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidases, human myeloperoxidase and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts (Fig. 1) (Stiborová et al., 2007). The same DNA adducts by ellipticine were also detected in human breast adenocarcinoma MCF-7 cells (Bořek-Dohalská et al., 2004), in V79

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Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (Frei et al., 2002), in leukemia HL-60 and CCRF-CEM cells (Poljaková et al., 2007) and *in vivo* in rats exposed to this anticancer drug (Stiborová et al., 2003a). On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues.

Because of the important role of CYP enzymes in ellipticine metabolism, expression levels of these enzymes are crucial for antitumor, cytostatic and/or genotoxic activities of this drug in individual tissues. Indeed, we found recently that the formation of the major ellipticine-derived DNA adduct (adduct formed in deoxyguanosine from 13-hydroxyellipticine, Fig. 1) in different organs of rats exposed to ellipticine is dependent on expression levels of CYP3A1. This is also the most efficient rat CYP enzyme activating ellipticine to form this DNA adduct *in vitro* (Stiborová et al., 2001; 2003a; c; Kotrbová et al., 2006). Likewise, the orthologous human enzyme, CYP3A4 in human liver microsomes, is also the most efficient enzyme activating ellipticine to 13-hydroxyellipticine and DNA adducts (Stiborová et al., 2004). In rats *in vivo*, however, not only CYP3A1, but also CYPs of the 1A subfamily, seem to be important for the formation of this adduct even though in *in vitro* incubations CYP1A enzymes are much less active than CYP3A1 (Stiborová et al., 2001; 2003a; c; Kotrbová et al., 2006). One of the reasons for this observed discrepancy might be the possibility of ellipticine-mediated induction of CYP1A enzymes in rats, which results in their higher protein levels and activities *in vivo*.

CYP1A1 and 1A2 enzymes are induced by a variety of compounds, of which dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are the most effective (Couture et al., 1990). Dioxins and related compounds induce CYP1A by binding to the ligand-activated transcription factor, aryl hydrocarbon receptor (AhR). Binding of ellipticine to this transcription factor has already been described (Fernadez et al., 1988), suggesting that this

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drug may induce CYP1A expression (Cresteil et al., 1982; Gasiewicz et al. 1996). Indeed, the previous studies of Cresteil et al. (1982) have shown that ellipticine is a CYP1A inducer, although these authors did not determine which members of this subfamily were affected. The present study was aimed at elucidating this question. Because rats were found to be suitable models mimicking the fate of ellipticine in humans (Stiborová et al., 2003a; c), they were used as model organisms in this study. Furthermore, since the constitutive and induced expression of rat CYP1A is sexually dimorphic (Iba et al., 1999), rats of both sexes were employed in some experiments.

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Materials and Methods

Chemicals. $NADP^+$, NADPH, ellipticine, D-glucose 6-phosphate, D-glucose 6-phosphate dehydrogenase and calf thymus DNA were obtained from Sigma Chemical Co (St Louis, MO, USA); Sudan I from BDH (Poole, UK); 7-ethoxyresorufin and 7-methoxyresorufin from Fluka Chemie AG (Buchs, Switzerland); 9-hydroxyellipticine (5,11-dimethyl-9-hydroxy-6*H*-pyrido[4,3-*b*]carbazole) were from Calbiochem (San Diego, CA, USA). All these and other chemicals from commercial sources used in the experiments were reagent grade or better. 7-Hydroxyellipticine and the N^2-oxide of ellipticine were synthesized as described (Wijsmuller et al. 1986; Boogaard et al. 1994) by J. Kučka (Charles University, Prague, Czech Republic); their purity was >99.5% as estimated by high-performance liquid chromatography (HPLC). Enzymatically prepared 12-hydroxy- and 13-hydroxyellipticine were obtained from multiple HPLC runs of ethyl acetate extracts of incubations of ellipticine with human and/or rat hepatic microsomes as described (Stiborová et al., 2004).

Animal Experiments. The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with Declaration of Helsinki. Male and female Wistar rats (~100 g) were treated with a single dose of 4, 40 or 80 mg/kg body weight (n=3) of ellipticine by intraperitoneal injection. Ellipticine was dissolved in sunflower oil/DMSO (1:1, v/v) to give a concentration of 6 mg/ml. Three control animals received an equal volume of solvent only. Rats were placed in cages in temperature and humidity controlled rooms. Standardized diet and water were provided *ad libitum*. The animals were killed 48 hours after treatment by cervical dislocation. Livers, lungs and kidneys were removed immediately after death and used for isolation of mRNA and for preparation of microsomal fractions. To analyze the duration of CYP induction, thirteen male rats were treated with 80 mg ellipticine per kg body weight in one dose; rats were sacrificed 2 days (3 animals), 2 weeks (2 animals), 10 weeks (3

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animals) and 32 weeks (5 animals) after the treatment by cervical dislocation and their livers used for isolation of microsomes.

Preparation of Microsomes. Microsomes were isolated from the livers, kidneys and lungs of rats as described (Stiborová et al., 2003b). Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with bovine serum albumin as a standard (Wichelman et al., 1988). The concentration of CYP was estimated according to Omura and Sato (1964) based on the absorption of the complex of reduced CYP with carbon monoxide. The total amounts of hepatic CYP are shown in Table 1. Kidney microsomes of control and ellipticine-treated rats (40 mg/kg) contained 0.17 and 1.19 nmol CYP/mg protein, respectively. Using the method of Omura and Sato (1964), no CYP was detectable in lung microsomes. Hepatic, renal and pulmonary microsomal preparations from rats that had been pre-treated with ellipticine were analyzed for the presence of ellipticine or its metabolites by HPLC as described (Stiborová et al., 2004). Neither ellipticine nor any of its metabolites were detectable in microsomal fractions from tissues of rats that had been pretreated with ellipticine.

Isolation of CYPs. CYP2B4 and 2E1 enzymes were isolated from liver microsomes of rabbits induced with phenobarbital (CYP2B4) or ethanol (CYP2E1) by procedures described (Stiborová et al., 2002a). Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA (Stiborová et al., 2002b), in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, Texas, USA) by P. Hodek (Charles University, Prague, Czech Republic). Human recombinant CYP3A4 was a gift of P. Anzenbacher (Palacky University, Olomouc, Czech Republic).

Preparation of antibodies. Leghorn chickens were immunized subcutaneously three times (at week interval) with CYP antigens (rat recombinant CYP1A1, rabbit CYP2B4, rabbit CYP2E1, human recombinant CYP3A4) (0.1 mg/animal) emulsified in complete Freund's

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adjuvant for the first injection and in incomplete adjuvant for boosters. Immunoglobulin fraction was isolated from pooled egg yolks using fractionation with polyethylene glycol 6000 (Stiborová et al., 2002b).

Determination of CYP Protein Levels in Microsomes of Rat Liver, Kidney and Lung. Immunoquantitation of rat liver, kidney and lung microsomal CYPs (CYP1A1, 1A2, 2B, 2E1 and 3A) was done by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Samples containing 75 μg microsomal proteins were subjected to electrophoresis on SDS/10% polyacrylamide gels (Stiborová et al., 2002b; 2005). After migration, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. CYP proteins were probed with the chicken polyclonal antibodies as reported elsewhere (Stiborová et al., 2002b; 2005; 2006). The antibody against rat recombinant CYP1A1 recognizes both CYP1A1 and 1A2 in rat liver microsomes (Figs. 2 and 3) (Stiborová et al., 2002b). Rat recombinant CYP1A1 and -1A2 (in Supersomes™, Gentest Corp., Woburn, MA, USA) were used as positive controls to identify the bands of CYP1A1 and 1A2 in microsomes. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as chromogenic substrate (Stiborová et al., 2002b; 2005; 2006).

CYP1A Enzyme Activity Assays. The microsomal samples were characterized for CYP1A activity using 7-ethoxyresorufin *O*-deethylation (EROD), CYP1A2 activity using 7-methoxyresorufin *O*-demethylation (MROD) (Guengerich and Shimada, 1991) and for the oxidation of Sudan I (a marker substrate for CYP1A1) (Stiborová et al., 2002b; 2005).

CYP1A mRNA Content in Rat Livers, Kidneys and Lungs. Total RNA was isolated from frozen livers, kidneys and lungs of three untreated rats and three rats pretreated with 40 mg/kg body weight of ellipticine using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the procedure supplied by the manufacturer. The quality of isolated RNA was

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verified by horizontal agarose gel electrophoresis and RNA quantity was assessed by UV-VIS spectrophotometry on a Carry 300 spectrophotometer (Varian, Palo Alto, CA, USA). RNA samples (1 μg) were reversely transcribed using 200 U of reverse transcriptase per sample with random hexamer primers utilizing RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The prepared cDNA was used for real-time (RT) polymerase chain reaction (PCR) performed in a RotorGene 2000 (Corbett Research, Sydney, Australia) under the following cycling conditions: incubation at 50 ºC for 2 min and initial denaturation at 95 ºC for 10 min, then 50 cycles of denaturation at 95 ºC for 15 s and annealing at 60 ºC for 1 min. Gain was set to 7 and fluorescence was acquired after elongation step. The PCR reaction mixtures (20 μl) contained 9 μl cDNA diluted 10-times in Milli-Q ultrapure water (Biocel A10, Millipore, Billerica, MA, USA), 10 μl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 1 μl TaqMan Gene Expression Assay Mix (commercially available unlabeled PCR primers and FAM™ dye-labelled probe for rat *CYP1A1/2* as target genes and *β-actin* as reference internal standard gene). Each sample was analysed in two parallel aliquots. Negative controls had the same composition as samples but cDNA was omitted from the mixture. Data were analysed by the program RotorGene v6 (Corbett Research, Sydney, Australia) and evaluated by comparative cycle threshold (c_T) method for relative quantitation of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then $\Delta\Delta c_T$ was evaluated according to the following equations: $\Delta c_T = c_T$ (target) - c_T (internal standard), $\Delta\Delta c_T = \Delta c_{Ttreated} - \Delta c_{Tcontrol}$, where $\Delta c_{Ttreated}$ is Δc_T for ellipticine treated rats and $\Delta c_{Tcontrol}$ is Δc_T for untreated rats. Δc_T is positive if the target is expressed at a lower level than the internal standard (*β-actin*), and negative if expressed at a higher level. The induction of mRNA expression of the studied target genes (fold change) in pretreated animals was evaluated as $2^{-(\Delta\Delta cT)}$.

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Microsomal Incubations. Incubation mixtures used to asses DNA adducts formed by ellipticine consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, pooled hepatic microsomal sample (0.5 mg protein) from 3 male rats, either control or treated with 4, 40 and 80 mg/kg body weight of ellipticine, 100 µM ellipticine (dissolved in 7.5 µl methanol) and 0.5 mg of calf thymus DNA in a final volume of 750 µl. The reaction was initiated by adding ellipticine. In incubations with lung and kidney microsomes control microsomes were compared with microsomes from rats treated with 40 mg/kg body weight. Incubations were carried out at 37°C for 30 min; ellipticine-DNA adduct formation was found to be linear up to 30 min of incubation (Stiborová et al., 2001). Control incubations were carried out either (i) without microsomes or (ii) without NADPH, (iii) without DNA or (iv) without ellipticine. After the incubation, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (Stiborová et al., 2001).

Incubation mixtures used to study the ellipticine metabolites contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase (NADPH-generation system), 0.2 mg protein of pooled hepatic microsomal fraction from 3 male rats, either untreated or treated with 40 mg/kg body weight ellipticine and 10 µM ellipticine (dissolved in 5 µl methanol) in a final volume of 500 µl. The reaction was initiated by adding the substrate. In the control incubation, ellipticine was omitted from the incubation mixture. After incubation in open glass tubes (37°C, 20 min) the reaction was stopped by adding 100 µl of 2 M NaOH. The oxidation of ellipticine is linear up to 30 min of incubation (Stiborová et al., 2004). After incubation, 5 µl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethyl acetate (2 x 1 ml). Analyzes of ellipticine metabolites were performed by HPLC as described (Stiborová et al., 2004). Recoveries of ellipticine metabolites were around 95% in the presence of microsomes without a CYP cofactor (NADPH-generation system).

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Inhibition studies. The following chemicals were used to inhibit the activation of ellipticine in hepatic microsomes of male rats: α-naphthoflavone (α-NF), which inhibits CYP1A1 and 1A2, being more efficient to inhibit CYP1A1 (Rendic and DiCarlo, 1997; Stiborová et al., 2005), furafylline, which inhibits CYP1A2, and troleandomycin and ketoconazole, which inhibit CYP3A (Rendic and DiCarlo, 1997). Inhibitors were dissolved in 7.5 μl of methanol, to yield final concentrations of 100 μM in the incubation mixtures used to asses DNA adducts formed by ellipticine (see above). Mixtures were then incubated at 37ºC for 10 min with NADPH prior to adding ellipticine, and then incubated for a further 30 min at 37ºC. After the incubation, DNA was isolated as mentioned above.

^{32}P-Postlabelling analysis and HPLC analysis of ^{32}P-labelled 3',5'-deoxyribonucleoside bisphosphate adducts. The ^{32}P-postlabeling of nucleotides using nuclease P1 enrichment procedure, found previously to be appropriate to detect and quantify ellipticine-derived DNA adducts formed *in vitro* (Stiborová et al., 2001; 2003c; 2004; 2007) and *in vivo* (Stiborová et al., 2003a), was employed in the experiments. The TLC and HPLC analyzes were done as reported recently (Stiborová et al., 2001; 2003a; c; 2004; 2007).

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Results

Effect of Ellipticine on CYP Expression. Western blots with chicken polyclonal antibodies raised against different CYPs (CYP1A1, 2B4, 2E1 and 3A4) showed that the expression of hepatic CYP1A1 and 1A2 were induced in male and female rats treated with ellipticine (Fig. 2). The expression of hepatic CYP2B, 2E1 and 3A was not altered by treating rats with ellipticine (data not shown).

Constitutive levels of CYP1A1 and 1A2, and their induction by ellipticine were sex-dependent**;** constitutive protein expression of CYP1A1 was higher in female rats, whereas that of CYP1A2 in males **(**Fig. 2). The increase in hepatic CYP1A1 and 1A2 expression by ellipticine was dose-dependent in all cases (Fig. 2) and correlated significantly with all doses of ellipticine administered to male rats [correlation coefficients of 0.807 ($p<0.05$, n=4) for CYP1A1 and 0.896 ($p<0.05$, n=4) for CYP1A2], but only for doses of 4 and 40 mg/kg body weight administered to female rats [correlation coefficients of 0.966 ($p<0.01$, n=3) for CYP1A1 and 0.981 ($p<0.01$, n=3) for CYP1A2]. Lower induction of CYP1A in female rats treated with 80 mg/kg body weight may be caused by a higher sensitivity of female rats to the toxicity of the administered compound. Indeed, ellipticine at doses above 40 mg/kg was found to be toxic to rats (Cresteil et al., 1982). The dose of 80 mg/kg also caused a decrease in levels of total hepatic CYP in female rats, but an increase in total CYP levels in male rats (Table 1).

The EROD activity, a marker for CYP1A1 and 1A2, the MROD activity, a marker for CYP1A2, as well as the oxidation of Sudan I, a marker substrate of CYP1A1 (Stiborová et al., 2002b; 2005), increased in hepatic microsomes of rats treated with ellipticine (Table 2).

Ellipticine also induced CYP1A1 levels and its activities in kidneys and lungs (Fig. 3). Male rats were employed in these experiments. Because of very low CYP1A1 protein levels and activities in kidneys and lungs of control animals, the ellipticine-mediated induction in these organs could not be quantified.

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In addition to evaluating the effects of ellipticine on protein levels and enzyme activities of CYP1A1 and 1A2, modulation of their mRNA expression by this compound in male rats was also investigated. As shown in Table 3, treatment of male rats with 40 mg/kg body weight of ellipticine induced an increase in mRNA expression levels of CYP1A1 in livers, kidneys and lungs, while no significant increase in CYP1A2 mRNA expression was observed.

Ellipticine-mediated CYP1A1 and 1A2 Induction is Transient. CYP1A1 and 1A2 protein expression was measured in livers of male rats 2 days, 2, 10 and 32 weeks after administration of a single i.p. dose of 80 mg ellipticine/kg body weight. Maximum CYP1A1/2 expression levels in this organ were found 2 days after treatment, the earliest time point at which liver samples were collected. As shown in Figure 4 a decline almost to the basal level of CYP1A1/2 protein expression and their enzymatic activities were detected 2 weeks after the dose.

Oxidation of Ellipticine by Rat Hepatic, Renal and Pulmonary Microsomes to DNA-Binding Metabolites. We compared the formation of adducts in calf thymus DNA by ellipticine incubated with microsomes from the livers, kidneys and lungs of treated and control male rats. The DNA adduct pattern generated by ellipticine consisted of at least two adducts (spots 1 and 2 in Figure 5), which were identical to those formed *in vivo* in rats treated with ellipticine (Fig. 5C), in *in vitro* incubations of DNA with ellipticine and human and rat hepatic microsomes (Stiborová et al., 2001; 2003a; 2004), and in those with 13-hydroxyellipticine (Fig. 5D) or 12-hydroxyellipticine (Fig. 5E). Lung and kidney microsomes were 22.4- and 32.7-fold less efficient than microsomes isolated from livers (Fig, 6; Supplemental data Tables S1 and S2).

Two additional minor ellipticine-derived DNA adducts (spots 6 and 7 in Fig. 5A,B) were found in DNA incubated with ellipticine and rat hepatic microsomes. These adducts are also formed *in vivo* (Fig. 5C) and in several *in vitro* activation systems, such as human liver

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microsomes (Stiborová et al., 2004) and peroxidases (Stiborová et al., 2007), but the low adduct levels prevented HPLC co-chromatographic analyses or their further characterization. Thin-layer chromatograms of ^{32}P-labelled DNA from control incubations carried out in parallel, without ellipticine or without DNA, were devoid of all adduct spots in the region of interest (data not shown). Control incubations without microsomes or NADPH were free of adduct spots 1, 6 and 7, but the adduct spot 2 was always detected (Supplemental data Table S1). This finding is consistent with our previous results showing that this adduct is formed also non-enzymatically (Stiborová et al., 2001; 2003c; 2004; 2007).

Ellipticine DNA adduct levels generated by liver, lung and kidney microsomes isolated from male rats treated with ellipticine were higher than DNA adduct levels formed by control microsomes (Fig. 6; Supplemental data Table S1). The increase in levels of DNA adducts formed in hepatic microsomes of rats treated with ellipticine might be attributed to an increase in enzymatic activity of both CYP1A1 and 1A2. While the relative contributions of both two CYP enzymes to the ellipticine-DNA adduct formation in hepatic microsomes of control rats was lower than those of CYP3A enzymes, in hepatic microsomes of rats pretreated with 40 mg/kg ellipticine, their contributions increased. This follows from the effect of inhibitors of CYP enzymes of both two subfamilies (Fig. 7). α-NF, an inhibitor of CYP1A1/2 with the predominant inhibitory effect on CYP1A1, and furafylline, an inhibitor of CYP1A2, decreased the levels of ellipticine-DNA adducts generated by hepatic microsomes of control rats by 62 and 50%, respectively, while troleandomycin and ketoconazole, selective inhibitors of CYP3A enzymes, were more effective. These two inhibitors decreased levels of ellipticine-DNA adducts by 75-92% (Fig. 7). This finding indicate that CYP3A enzymes play a major role in ellipticine-DNA adduct formation in livers of control (uninduced) rats. On the contrary, the effect of CYP1A inhibitors in hepatic microsomes of rats pretreated with

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ellipticine was up to 4-times higher than in those of control animals, being more pronounced for α-NF than for furafylline (Fig. 7, Supplemental data Table S3).

The increase in levels of ellipticine-DNA adducts correlates with an increase in ellipticine oxidation by hepatic microsomes from the 40 mg ellipticine/kg group (Figs. 6 and 8). Besides an increase in oxidation of ellipticine to 9-hydroxyellipticine and 7-hydroxyellipticine, which was expected, because these metabolites are predominantly formed by CYP1A1/2 (Stiborová et al., 2004; Kotrbová et al., 2006), up to a 2-fold increase in formation of 13-hydroxy- and 12-hydroxyellipticine, the metabolites generating DNA adducts 1 and 2, was found (Fig. 8).

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Discussion

Ellipticine is an anticancer agent, whose biological effects such as pharmacological efficiencies and its potential genotoxic side effects depend on its CYP-mediated metabolism (Stiborová, et al. 2001; 2003a; 2004). Although the CYPs responsible for the activation of this drug to DNA binding species as well as those detoxifying this compound have already been identified (Stiborová et al., 2003a; 2004), knowledge about the effects of ellipticine exposure on the expression and activities of these enzymes has, until now, been limited.

In the present study, we have found that expressions of CYP1A1 and/or 1A2 proteins as well as their enzymatic activities were significantly induced by ellipticine in livers, lung and kidneys of Wistar rats treated with ellipticine. This induction might be a consequence of the ellipticine-binding to AhR described by several authors (Fernadez et al., 1988; Gasiewicz et al., 1996). Ellipticine binding allows the cytosolic AhR to translocate into the nucleus and to dimerize with AhR nuclear translocator (ARNT). The AhR-ARNT complex functions as a transcriptional activator by binding to the Ah responsive element in the regulatory domains of *CYP1A1* and *CYP1A2* genes (Gasiewicz et al., 1996), thus stimulating their transcription (present paper).

Another mechanism of CYP1A1 induction might result from the inhibition of the oxidation of other substrates of this enzyme by ellipticine (Aimová and Stiborová, 2005). As shown by Chang and Puga (1998), a decrease in oxidation of an endogenous substrate of CYP1A1 by ellipticine results in an increase in constitutive activation of AhR-ARNT transcriptional complexes. In addition, low levels of ellipticine antagonize AhR activation by inducing ligands such as TCDD.

Differences in constitutive levels and inducibility of CYP1A1 and 1A2 by ellipticine were found in male and female rats. The induction of CYP1A1/2 proteins by ellipticine, when expressed as fold over control values, is higher in males than in females. Similar results in

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constitutive levels and inducibility of CYP1A1/2 by β-naphtoflavone (β-NF) were found in Sprague-Dawley rats (Iba et al., 1999); males were more responsive than females to induction by β-NF. However, the absolute levels of CYP1A1/2 protein at 40 mg/kg ellipticine appear similar in male and female rat liver (see immunodetection of these CYPs shown in Figure 2). Therefore, the relative induction of CYP1A depends on the level of the controls. The CYP1A induction by 80 mg/kg body weight in females was lower than that by the 40 mg/kg ellipticine dose. The question whether a higher sensitivity of female rats to the toxicity of ellipticine (Cresteil et al., 1982) may be the reason for this observation remains to be answered. Another sex-dependent difference, which awaits further investigation, was found for the CYP1A-selective activities catalyzed by hepatic microsomes. Whilst there is a good agreement in fold-induction between CYP1A2 protein and MROD in males and females, this relationship holds only in females for CYP1A1 protein and Sudan I oxidation. In males, fold-induction is much greater for CYP1A1 protein, perhaps reflecting the lower accuracy of the measurement in the control animals.

The CYP1A induction by ellipticine resulted in up to 3.8-fold higher ellipticine-DNA adduct levels in incubations of ellipticine with microsomes from rats treated with this compound than in incubations with control microsomes. This increase corresponded to an increase in ellipticine oxidation to 13-hydroxy- and 12-hydroxyellipticine, the metabolites generating the two major DNA adducts. However, the increased ellipticine-DNA adduct formation caused by induction of CYP1A enzymes was 9- and 1.5-times lower than the increase in CYP1A1 and 1A2 protein levels and was also lower than the increase in CYP1A1- and/or 1A2-mediated activities. These differences might be explained by the efficiencies of induced CYP1A1/2 to oxidize ellipticine not only to 13-hydroxy- and 12-hydroxyellipticine, but also to the metabolites that do not form DNA adducts, namely, to 9-hydroxy- and 7-hydroxyellipticine (Stiborová, et al., 2004).

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The results found in this study, demonstrating that ellipticine-mediated induction of CYP1A leads to an increase in ellipticine activation to species forming DNA adducts, shed light on our previous data, showing the importance of CYP1A enzymes in ellipticine activation *in vivo* (Stiborová et al., 2003c). The importance of CYP1A in ellipticine-derived DNA adduct formation *in vivo* was initially rather surprising for us, because it did not correspond to the *in vitro* situation (Stiborová et al., 2001; 2003a). 13-Hydroxyellipticine, the metabolite responsible for generation of adduct spot 1 due to the formation of the reactive species ellipticine-13-ylium (Fig. 1), was found to be formed by CYP1A1/2 *in vitro* only in low amounts (Stiborová et al., 2004). Here, we show that one of the reasons for the *in vivo* situation might be the induction of CYP1A with ellipticine; in hepatic microsomes from rats induced with ellipticine higher levels of ellipticine-DNA adducts were generated. Indeed, the relative contributions of CYP1A (predominantly CYP1A1) to the formation of ellipticine-DNA adducts were increased in hepatic microsomes of induced rats.

The induction of CYP1A by ellipticine is transient. In the absence of repeated administration of ellipticine, the amount and activities of the induced CYP1A decreased almost to the basal level two weeks after treatment.

The induction of CYP1A1 protein expression and enzymatic activities by ellipticine corresponded to elevated mRNA levels of this enzyme. Levels of CYP1A2 mRNA were, however, unaffected despite the increase in both protein and activity. Similar discrepancies between induction of CYP1A mRNAs and protein levels were observed previously (Dagawa et al., 1998; Chen et al., 1998; Dickins, 2004; Stiborová et al., 2006). Detailed analyses of the time-dependence of the expression levels of mRNAs and proteins of the tested enzymes were not performed in this study, but they might answer the questions whether the transient induction of the mRNAs of CYP1A, or the different half-lives for their mRNAs and proteins,

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and/or the effects of ellipticine on the stability of mRNAs and proteins of these enzymes are the rationale for our observation.

The rat was used as an experimental model on the basis that the results should provide some indication of what might occur in patients treated with the drug. However, it should be noted that the similarities as well as differences in the CYP1A systems and their induction exist in the two species. For example, in rats CYP1A1 is often more markedly induced in liver than CYP1A2 whereas the reverse is true for humans (Dickins, 2004). Furthermore, the specificity of rat and human CYP1A1 and 1A2 is known to differ, particularly for inhibitors (Guengerich and Shimada, 1991; Stiborová et al., 2001).

Recently, we have demonstrated that ellipticine is oxidatively activated to species binding to DNA also in human cancer cells such as the breast adenocarcinoma MCF-7 (Bořek-Dohalská et al., 2004) and leukemia HL-60 and CCRF-CEM cells (Poljaková et al., 2007). CYP1A1 is expressed in all these cells and was found to be one of the enzymes responsible for ellipticine-DNA binding and its cytotoxicity against these cancer cells (Bořek-Dohalská et al., 2004; Poljaková et al., 2007). Likewise, Rekha and Sladek (1997) demonstrated that the cytotoxic activity of ellipticine to MCF-7 cells depends on the levels of CYP1A enzymes. The cited authors showed that MCF-7 cells treated with another CYP1A inducer, 3-methylcholanthrene, transiently expressed elevated levels of CYP1A and were transiently much more sensitive to ellipticine. The DNA adducts we have observed in MCF-cells might be responsible for the higher sensitivity observed by the above authors.

Another important feature related to the expression of CYP1A and ellipticine toxicity was detected in MCF-7 cells selected for resistance to adriamycin (Adr^R MCF-7), exhibiting the phenotype of multidrug resistance (MDR) (Ivy et al., 1988). Ivy et al. (1988) postulated that the resistance of Adr^R MCF-7 cells involves several biochemical and genetic changes besides MDR. One of them is a regulatory defect at the level of CYP1A1 mRNA resulting in lower

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CYP1A1-mediated metabolism of xenobiotics in these cells. Adr^R MCF-7 cells are cross-resistant to ellipticine (Ivy et al., 1988), which we would explain by a decrease in the CYP1A1-dependent activation of ellipticine.

Another CYP enzyme expressed in MCF-7 cells and breast cancer, CYP1B1 (Shehin et al., 2000), is known to activate ellipticine, and transcriptional activation of *Cyp1b1* is believed to involve the AhR (Kerzee and Ramos, 2001). Therefore, its induction by ellipticine should also be evaluated. Taken together, the data in these studies indicate that the expression levels, activities and induction of CYP1A1 and 1B1, the enzymes activating ellipticine, may be an important factor in the specificity and efficiency of ellipticine for breast cancer. In this context, it will be important to evaluate whether ellipticine induces CYP1A1 and 1B1 expression in MCF-7 cells. Therefore, the study investigating the induction of CYP1A1 and 1B1 expression and their activities by ellipticine and their efficiencies to activate this chemical in MCF-7 cells is under way in our laboratory.

In conclusion, the results of the present study show for the first time that ellipticine is capable to induce CYP1A in rat livers, kidneys and lungs, an enzyme which is involved in ellipticine biotransformation in both rats and humans. By such effects, ellipticine might modulate its own disposition, pharmacological efficiency and/or genotoxic potential. Nevertheless, this feature has to be established *in vivo*.

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This article has not been copyedited and formatted. The final version may differ from this version.

References

Aimová D, and Stiborová M (2005) Antitumor drug ellipticine inhibits the activities of rat hepatic cytochromes P450. *Biomed Pap*, **149**: 437-440.

Auclair C (1987) Multimodal action of antitumor agents on DNA: The ellipticine series. *Arch Biochem Biophys,* **259**: 1-14.

Boogaard AT, Pandit UK, and Koomen GJ (1994) Ring-D modifications of ellipticine. 2. Chlorination of ellipticine via its *N*-oxide and synthesis and selective acetylation of 5,6,11-trimethyl-5*H* benzo[*B*]carbazole. *Tetrahedron* **50**: 4811-4828.

Bořek-Dohalská L, Frei E, and Stiborová M. (2004) DNA adduct formation by the anticancer drug ellipticine and its hydroxy derivatives in human breast adenocarcinoma MCF-7 cells. *Collect Czech Chem Commun* **69**: 603-615.

Chang C-Y, and Puga A (1998) Constitutive activation of the aromatic hydrocarbon receptor. *Mol Cell Biol* **18**: 525-535.

Chen RM, Chou MW, and Ueng TH (1998) Induction of cytochrome P450 1A in hamster liver and lung by 6-nitrochrysene. *Arch Toxicol* **72**: 395-401.

Couture L.A., Abbott B.D., and Birnbaum L.S. (1990) A critical review of the developmental toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: recent advances towards understanding the mechanism. *Teratology* 42: 619-627.

Cresteil T, Le Provost E, Leroux JP, and Lesca P (1982) Enzymatic and immunological characterization of cytochromes P 450 from ellipticine-treated rat liver. *Biochem Biophys Res Commun* **107**: 1037-1045.

Dagawa M, Nakayama M, Konno Y, Masobuchi K, and Yamazou Y (1998) 2-Methoxy-4-nitroaniline and its isomers induce cytochrome P450 1A (CYP1A) enzymes with different selectivities in the rat livers. *Biochim Biophys Acta* **1379**: 391-398.

Dickins M. (2004) Induction of cytochromes P450. *Curr Top Med Chem* **4**: 1745-1766.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 20, 2024

DMD Fast Forward. Published on July 26, 2007 as DOI: 10.1124/dmd.107.016048
This article has not been copyedited and formatted. The final version may differ from this version.

Fernandez N, Roy M, and Lesca P (1988) Binding characteristics of *Ah* receptors from rats and mice before and after separation from hepatic cytosols. 7-Hydroxyellipticine as a competitive antagonist of cytochrome P-450 induction. *Eur J Biochem* **172**: 585-592.

Fossé P, René B, Charra M, Paoletti C, and Saucier JM (1992) Stimulation of topoisomerase II-mediated DNA cleavage by ellipticine derivatives: structure-activity relationships. *Mol Pharmacol* 4**2**: 590-595.

Frei E, Bieler CA, Arlt VM, Wiessler M, and Stiborová M (2002) Covalent binding of the anticancer drug ellipticine to DNA in V79 cells transfected with human cytochrome P450 enzymes. *Biochem Pharmacol* 64: 289-295.

Froelich-Ammon SJ, Patchan MW, Osheroff N, and Thompson RB (1995) Topoisomerase II binds to ellipticine in the absence or presence of DNA. Characterization of enzyme-drug interactions by fluorescence spectroscopy. *J Biol Chem* **270**: 14998-5004.

Gasiewicz TA, Kende RS, Rucci G, Whitney B, and Willey JJ (1996) Analysis of structural requirements for Ah receptor antagonist activity: Ellipticines, flavones, and related compounds. *Biochem Pharmacol* **52**:1787-1830.

Guengerich FP, and Shimada T (1991) Oxidation of toxic and carcinogenic chemicals by human cytochrome P450 enzymes. *Chem Res Toxicol* **4**: 391-407.

Iba MM, Fung J,Thomas PE, and Park Y (1999) Constitutive and induced expression by pyridine and β-naphthoflavone of rat CYP1A is sexually dimorphic. *Arch Toxicol* **73**: 208-216.

Ivy SP, Tulpule A, Fairchild CR, Averbuch SD, Myers CE, Nebert DW, Baird WM, and Cowan KH (1988) Altered regulation of P-450IA1 expression in a multidrug-resistant MCF-7 human breast cancer cell line. *J Biol Chem* **26**: 19119-19125.

Kerzee J.K., and Ramos K.S. (2001) Constitutive and inducible expression of *Cyp1a1* and *Cyp1b1* in vascular smooth muscle cells. *Circ Res* **89**: 573-582.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 20, 2024

DMD Fast Forward. Published on July 26, 2007 as DOI: 10.1124/dmd.107.016048
This article has not been copyedited and formatted. The final version may differ from this version.

Kotrbová V, Aimová D, Březinová A, Janouchová K, Poljaková J, Hodek P, Frei E, and Stiborová M (2006) Cytochromes P450 reconstituted with NADPH:P450 reductase mimic the activating and detoxicating metabolism of the anticancer drug ellipticine in microsomes. *Neuro Endocrinol Lett* **27** (Suppl. 2): 18-20.

Omura T, and Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239:** 2370-2378.

Poljaková J, Frei E, Gomez JE, Aimová D, Eckschlager T, Hraběta J, and Stiborová M (2007) DNA adduct formation by the anticancer drug ellipticine in human leukemia HL-60 and CCRF-CEM cells. *Cancer Lett* **252**: 270-279.

Rendic S, and DiCarlo FJ (1997) Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev* **29**:413-480.

Rekha GK, and Sladek NE (1997) Multienzyme-mediated stable and transient multidrug resistance and collateral sensitivity induced by xenobiotics. *Cancer Chemother Pharmacol* **40**: 215-224.

Schwaller MA, Allard B, Lescot E, and Moreau F (1995) Protonophoric activity of ellipticine and isomers across the energy-transducing membrane of mitochondria. *J Biol Chem* **27**0: 22709-22713.

Shehin SE, Stephenson RO, and Greenleee WF (2000) Transcriptional regulation of the human CYP1B1 gene: evidence for involvement of an aryl hydrocarbon receptor response element in the constitutive expression. *J Biol Chem* **275**: 6770-6775.

Singh MP, Hill GC, Peoch D, Rayner B, Inabach JL, and Lown JW (1994) High-field NMR and restrained molecular modeling studies on a DNA heteroduplex containing a modified apurinic abasic site in the form of covalently linked 9-aminoellipticine. *Biochemistry* **33**: 10271-10285.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 20, 2024

DMD Fast Forward. Published on July 26, 2007 as DOI: 10.1124/dmd.107.016048
This article has not been copyedited and formatted. The final version may differ from this version.

Stiborová M, Bieler CA, Wiessler M, and Frei E (2001) The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts. *Biochem Pharmacol* **62**: 675-84.

Stiborová M, Bořek-Dohalská L, Hodek P, Mráz J, and Frei E (2002a) New selective inhibitors of cytochromes P450 2B and their application to antimutagenesis of tamoxifen. *Arch Biochem Biophys* **403**, 41-49.

Stiborová M, Breuer A, Aimová D, Stiborová-Rupertová M, Wiessler M, and Frei E (2003a) DNA adduct formation by the anticancer drug ellipticine in rats determined by ^{32}P-postlabeling. *Int J Cancer* **107**: 885-890.

Stiborová M, Dračínská H, Hájková J, Kadeřábková P, Frei E, Schmeiser HH, Souček P, Phillips DH, and Arlt VM (2006) The environmental pollutant and carcinogen 3-nitrobenzanthrone and its human metabolite 3-aminobenzanthrone are potent inducers of rat hepatic cytochromes P450 1A1 and -1A2 and NAD(P)H:quinone oxidoreductase. *Drug Metab Dispos* **34**: 1398-1405.

Stiborová M, Frei E, Sopko B, Sopková K, Marková V, Laňková M, Kumstýřová T, Wiessler M, and Schmeiser HH (2003b) Human cytosolic enzymes involved in the metabolic activation of carcinogenic aristolochic acid: evidence for reductive activation by human NAD(P)H:quinone oxidoreductase. *Carcinogenesis* **24**: 1695-1703.

Stiborová M, Martínek V, Rýdlová H, Hodek P, and Frei E (2002b) Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes. *Cancer Res* **62**: 5678-5684.

Stiborová M, Martínek V, Rýdlová H, Koblas T, and Hodek P (2005) Expression of cytochrome P450 1A1 and its contribution to oxidation of a potential human carcinogen 1-phenylazo-2-naphthol (Sudan I) in human livers. *Cancer Lett* **220**: 145-154.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 20, 2024

DMD Fast Forward. Published on July 26, 2007 as DOI: 10.1124/dmd.107.016048
This article has not been copyedited and formatted. The final version may differ from this version.

Stiborová M, Poljaková J, Ryšlavá H, Dračínský M, Eckschlager T, and Frei E. (2007) Mammalian peroxidases activate anticancer drug ellipticine to intermediates forming deoxyguanosine adducts in DNA identical to those found *in vivo* and generated from 12-hydroxyellipticine and 13-hydroxyellipticine. *Int J Cancer* **120**: 243-251.

Stiborová M, Sejbal J, Bořek-Dohalská L, Aimová D, Poljaková J, Forsterová K, Rupertová M, Wiesner J, Hudeček J, Wiessler M, and Frei E. (2004) The anticancer drug ellipticine forms covalent DNA adducts, mediated by human cytochromes P450, through metabolism to 13-hydroxyellipticine and ellipticine N^2-oxide. *Cancer Res* **64**: 8374-8380.

Stiborová M, Stiborová-Rupertová M, Bořek-Dohalská L, Wiessler M, and Frei E (2003c) Rat microsomes activating the anticancer drug ellipticine to species covalently binding to deoxyguanosine in DNA are a suitable model mimicking ellipticine bioactivation in humans. *Chem Res Toxicol* **16**: 38-47.

Sugikawa E, Hosoi T, Yazaki N, Gamanuma N, Nakanishi N, and Ohashi M (1999) Mutant p53 mediated induction of cell cycle arrest and apoptosis at G1 phase by 9-hydroxyellipticine. *Anticancer Res* **19**: 3099-3108.

Wiechelman KJ, Braun RD and Fitzpatrick JD (1988) Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Anal Biochem* **175:** 231-237.

Wijsmuller WFA, Warner MJ, Koonen GI, and Pandit UK (1986) Pyridocarbazole alkaloids. Synthesis of olivacine and ellipticine analogues. *Heterocycles* **24**: 1795-1797.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 20, 2024

DMD Fast Forward. Published on July 26, 2007 as DOI: 10.1124/dmd.107.016048
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Footnotes

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Legends for figures

FIG. 1. Metabolism of ellipticine by peroxidases and human CYPs showing the identified metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and are the electrophilic metabolites postulated as ultimate arylating species.

FIG. 2. Induction of CYP1A1 (A,B) and 1A2 (C,D) in livers of male (A,C) and female rats (B,D) treated with 4, 40 or 80 mg/kg of body weight ellipticine. Mean values ± standard deviations shown in figure represent results obtained from livers of three rats (n=3). Inset in A and B: immunoblots of microsomal CYP1A1 and 1A2 from untreated and ellipticine-treated male and female rats, respectively, stained with antibody against rat CYP1A1. Microsomes isolated from rat livers were subjected to SDS-PAGE, proteins transferred to PVDF membranes and probed with antibody as described in Material and Methods. Values significantly different from the control: $*p<0.05$, $**p<0.01$, $***p<0.001$ (Student's t-test).

FIG. 3. Immunoblots of microsomal CYP1A1 and 1A2 from livers, lungs and kidneys of untreated and ellipticine-treated (40 mg/kg) male rats stained with antibody against rat CYP1A1. Microsomes isolated from rat organs were subjected to SDS-PAGE, proteins transferred to PVDF membranes and probed with antibody as described in Material and Methods.

FIG. 4. Time course of ellipticine-mediated induction of CYP1A1 (A) and 1A2 protein (B) and specific CYP1A activities (C-D) in livers of male rats treated intraperitoneally with a single dose of 80 mg/kg body weight ellipticine. Results represent means ± standard deviations from three to five treated rats (see Materials and Methods). Inset: immunoblots of microsomal CYP1A1 and 1A2 from untreated and ellipticine-treated male rats stained with antibody against rat CYP1A1.

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FIG. 5. Autoradiographic profile of DNA adducts generated in calf thymus DNA by ellipticine after its activation with hepatic microsomes of untreated (A) and ellipticine-treated (40 mg/kg) male rats (B), of ^{32}P-labeled digests of DNA from liver of male rats treated with the same dose of ellipticine (C), from calf thymus DNA reacted with 13-hydroxyellipticine (D) or 12-hydroxyelipticine (E). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay.

FIG. 6. DNA adduct formation by ellipticine activated with microsomes isolated from livers, lungs and kidneys of male rats, control (uninduced) or pretreated with ellipticine as indicated. Mean RAL (relative adduct labelling) ± standard deviations shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent *in vitro* incubations). ND, not determined. Values significantly different from control: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ (Student's t-test).

FIG. 7. Effect of CYP1A and 3A inhibitors on ellipticine-DNA adduct formation in hepatic microsomes of untreated (A) and ellipticine-treated (40 mg/kg) male rats (B). Mean RAL ± standard deviations shown in the figure represent total levels of DNA adducts of three parallel *in vitro* incubations.

FIG. 8. Ellipticine metabolism in male rat hepatic microsomes of control animals and those treated with ellipticine. Microsomes containing 0.2 mg microsomal protein, and 10 μM ellipticine were used in all experiments. Levels of ellipticine metabolites are averages ± standard deviations of triplicate incubations. Values significantly different from control: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ (Student's t-test).

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TABLE 1

Total contents of CYP in hepatic microsomes of ellipticine-treated and control rats

Ellipticine dose (mg/kg)	CYP[a] in hepatic microsomes of male rats	female rats
	nmol/mg protein	
0	0.32 ± 0.07	0.42 ± 0.14
4	0.33 ± 0.03	0.32 ± 0.05
40	0.35 ± 0.06	0.34 ± 0.06
80	0.61 ± 0.08*	0.21 ± 0.02*

[a]Results shown are mean ± SD from data obtained by the method of Omura and Sato (1964) for three rats.

*Significantly different from controls: $p<0.01$ (Student's t-test).

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TABLE 2

Specific CYP1A activities [a] *in hepatic microsomes of control and ellipticine-treated rats*

CYP activity	Control rat		Ellipticine-treated rat	
	Male	Female	Male	Female
EROD	80.7 ± 2.0	225.8 ± 50.5	551.4 ± 92.2 (**6.9**)*	1737.5 ± 161.3 (**7.7**)*
MROD	3.28 ± 0.15	2.48 ± 0.24	19.73 ± 0.08 (**6.0**)*	20.07 ± 1.51 (**8.0**)*
Sudan I oxidation	0.125 ± 0.013	0.107 ± 0.010	1.463 ± 0.123 (**12.2**)*	1.491 ± 0.151 (**14.0**)*

[a]Each value (pmol of reaction product/min/nmol CYP) represents the mean ± standard deviation of data from two rats in two separate assays (n=4). Numbers in parentheses represents the fold increase over the control activity caused by the pre-treatment with ellipticine (40 mg/kg).

*Significantly different from controls: $p<0.001$ (Student's t-test).

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TABLE 3

Expression of mRNA of CYP1A1 and 1A2

	CYP1A1		CYP1A2	
	Δc_T[a]	Fold Change	Δc_T	Fold Change
Control rats				
Liver	6.37 ± 0.07	-	-3.90 ± 0.12	-
Kidney	4.45 ± 0.36	-	14.07 ± 0.54	-
Lung	10.48 ± 0.16	-	13.97 ± 1.06	-
Ellipticine-treated rats				
Liver	3.87 ± 1.73	5.66*	-4.04 ± 0.22	1.10
Kidney	1.12 ± 0.13	10.08**	13.80 ± 0.76	1.21
Lung	5.89 ± 0.59	24.20**	15.37 ± 0.03	0.38

[a]Results shown are mean ± standard deviation from data found for three male rats (control and treated with 40 mg/kg body weight).

Significantly different from controls: $^{*}p<0.05$; $^{**}p<0.001$ (Student's t-test).

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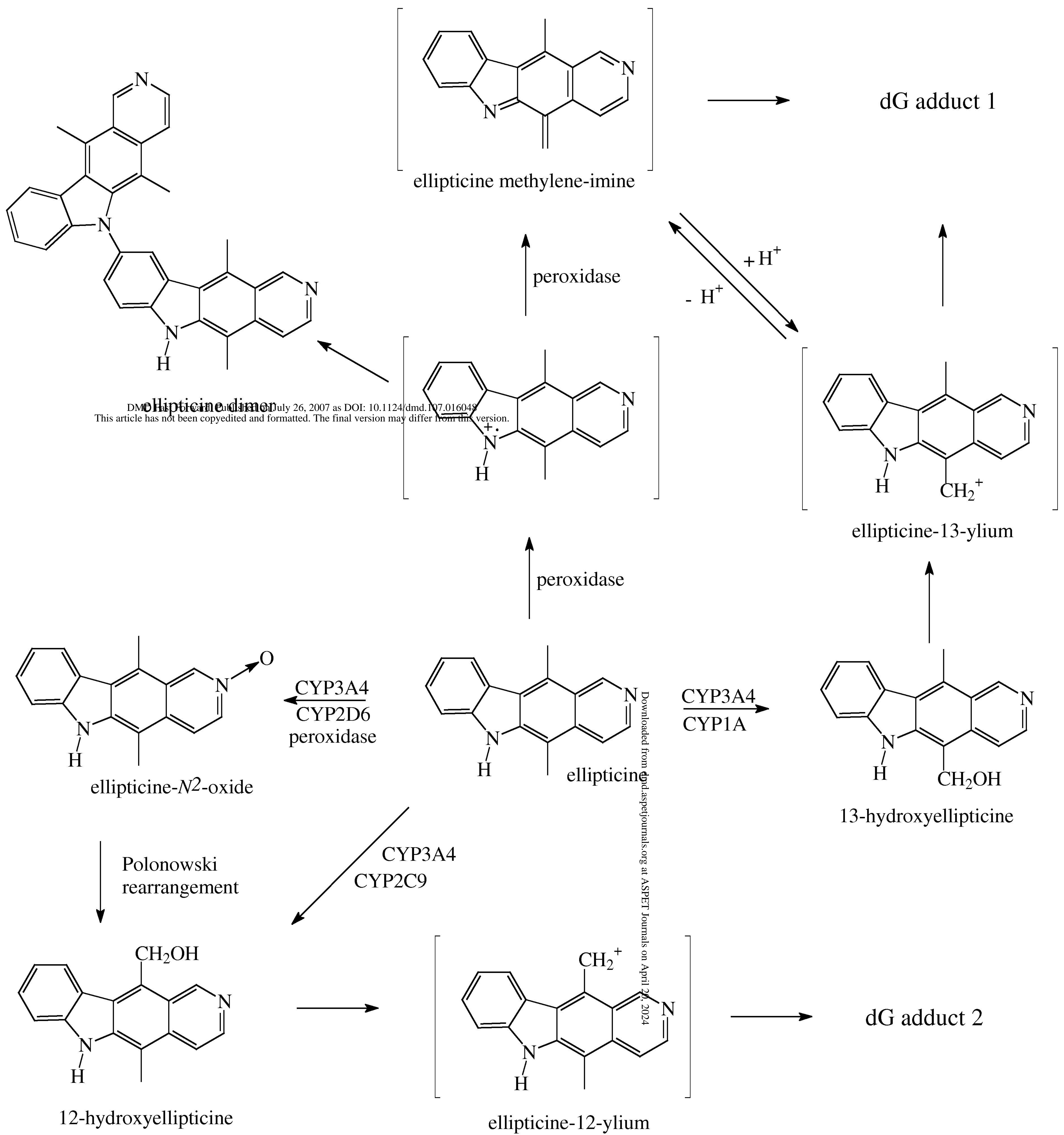

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Figure 1

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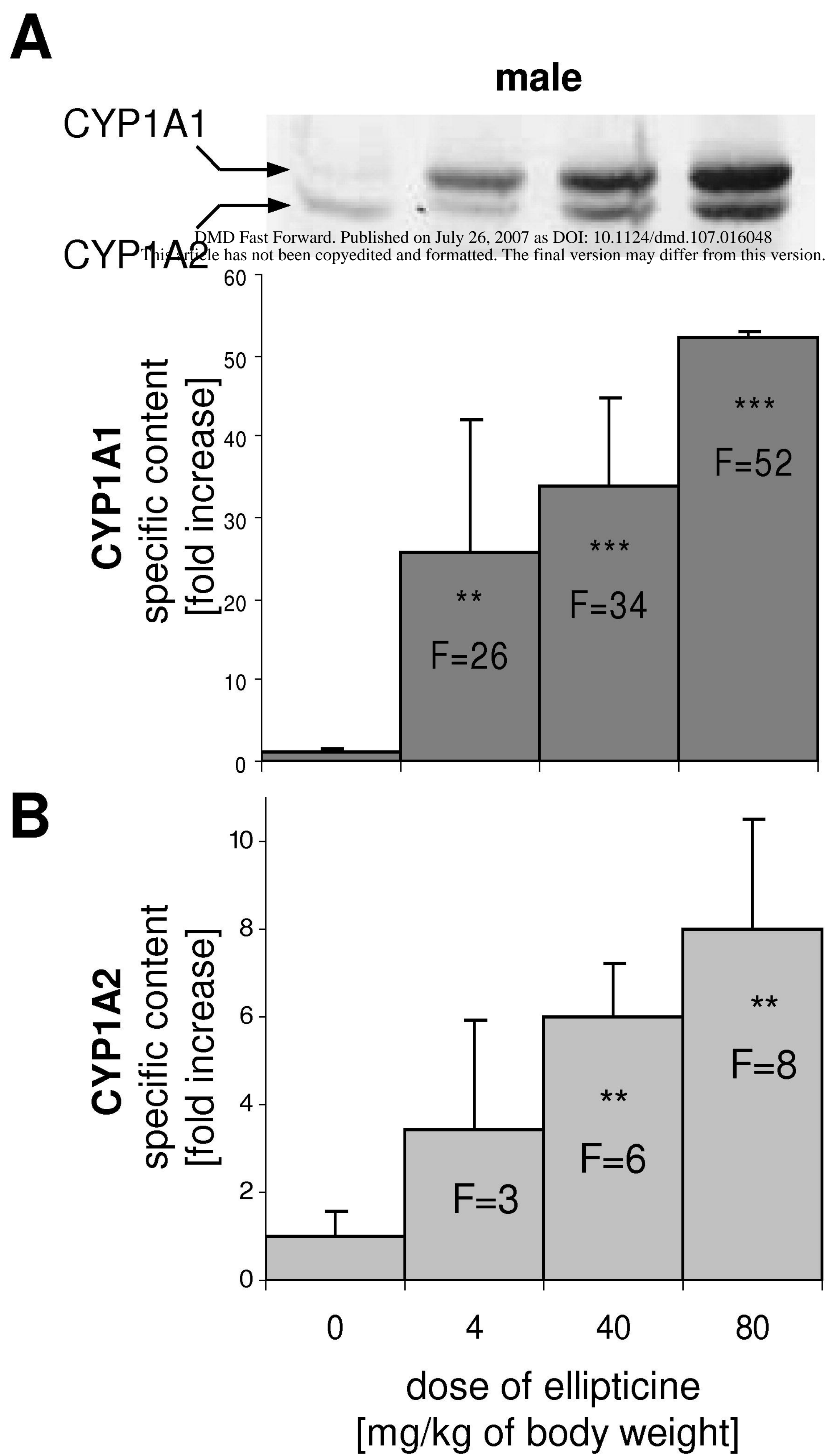

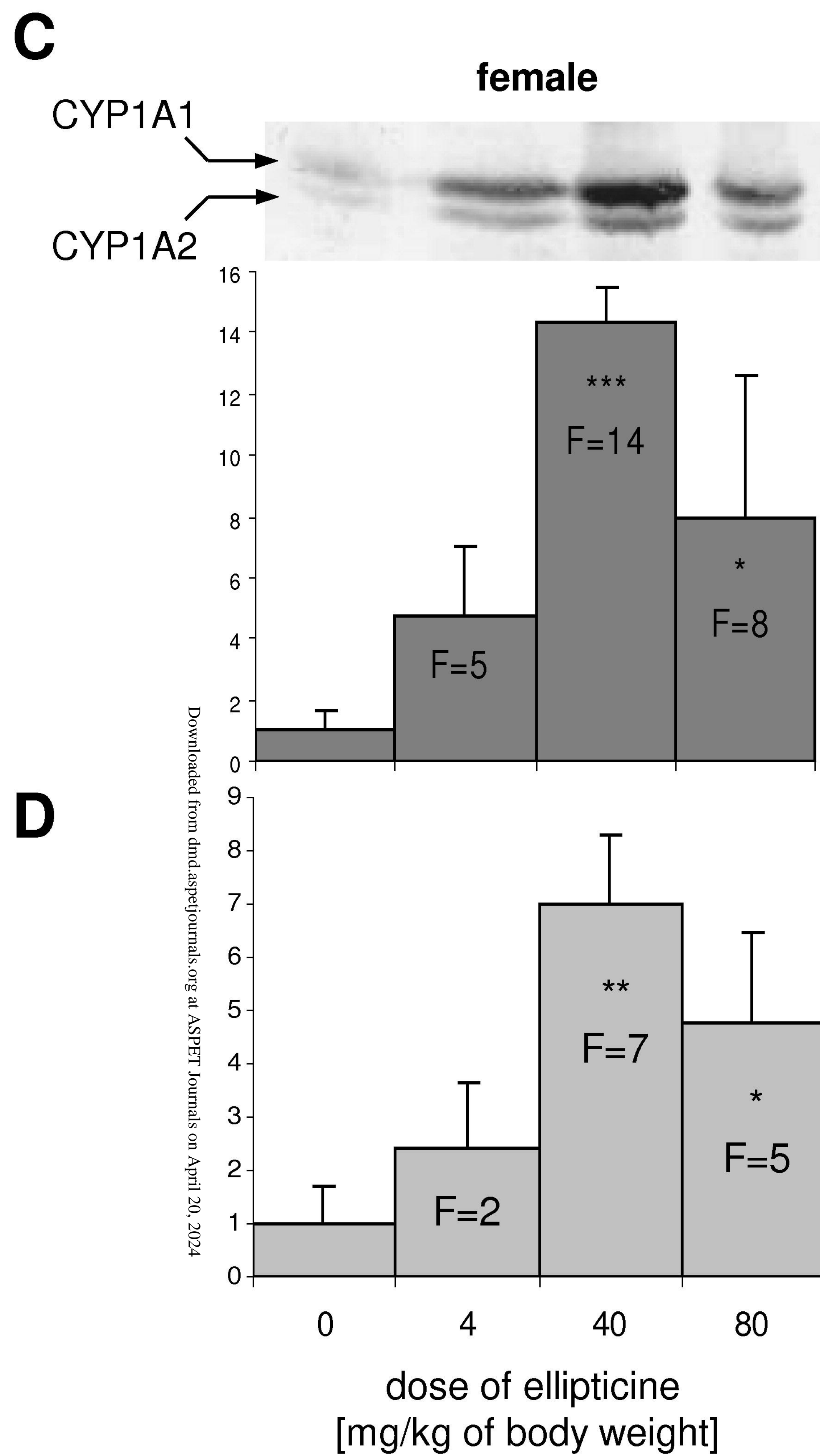

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Figure 2

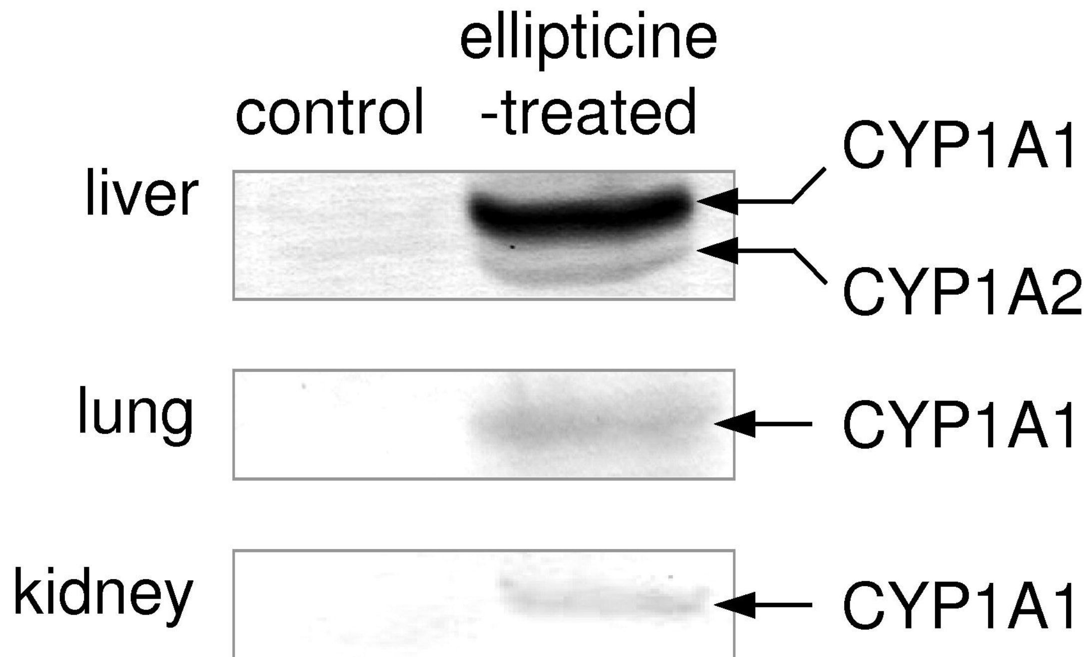

Figure 3

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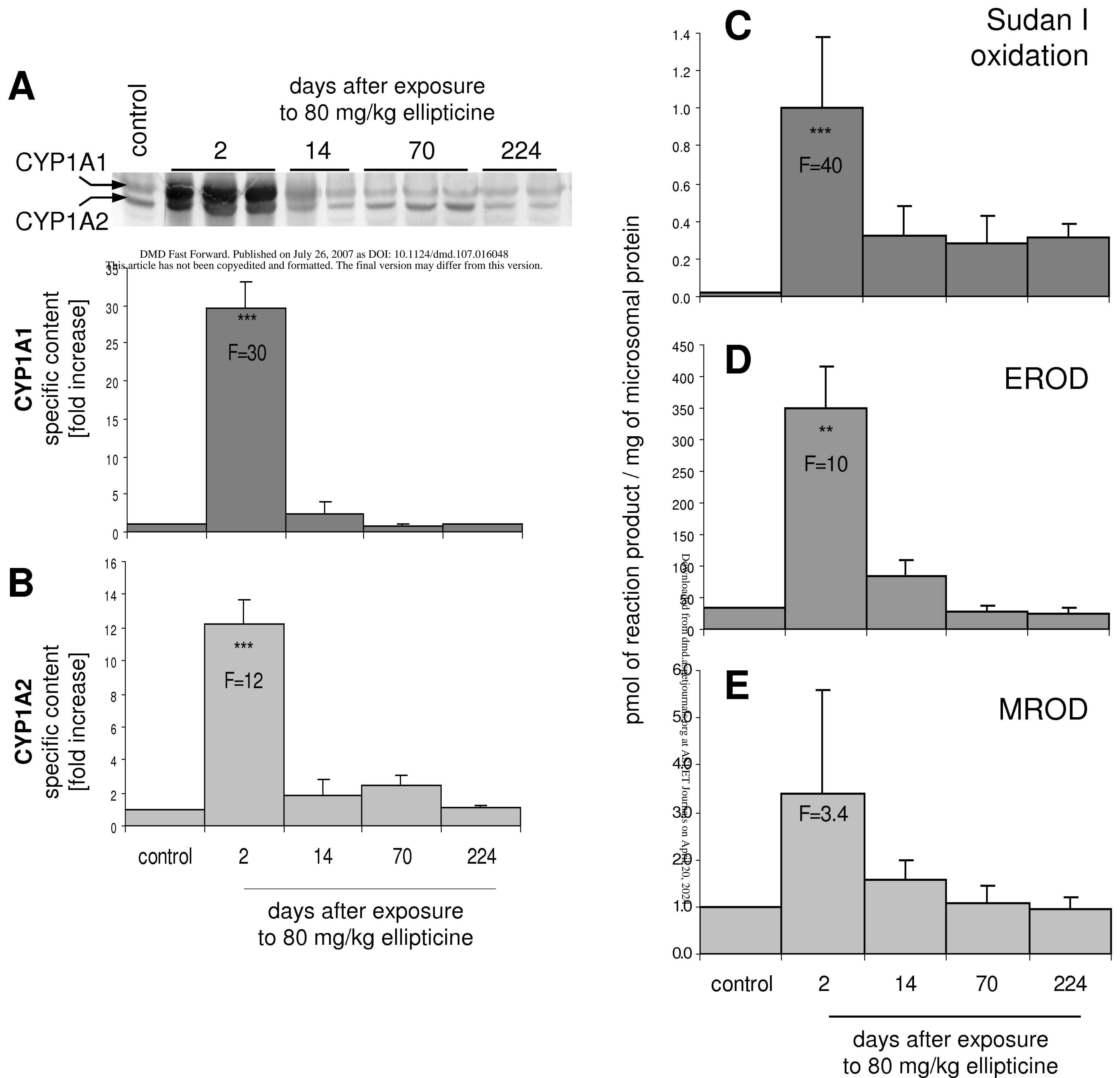

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Figure 4

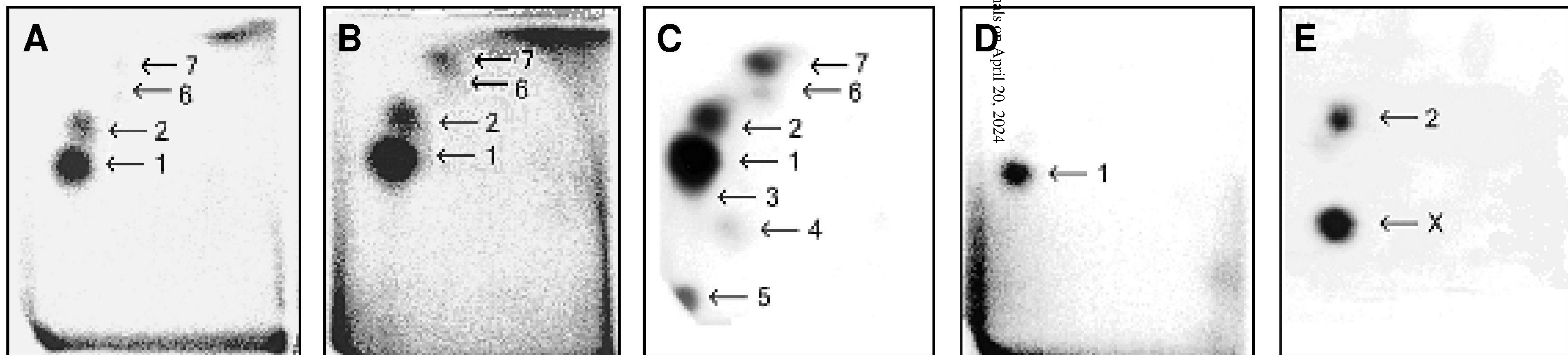

Figure 5

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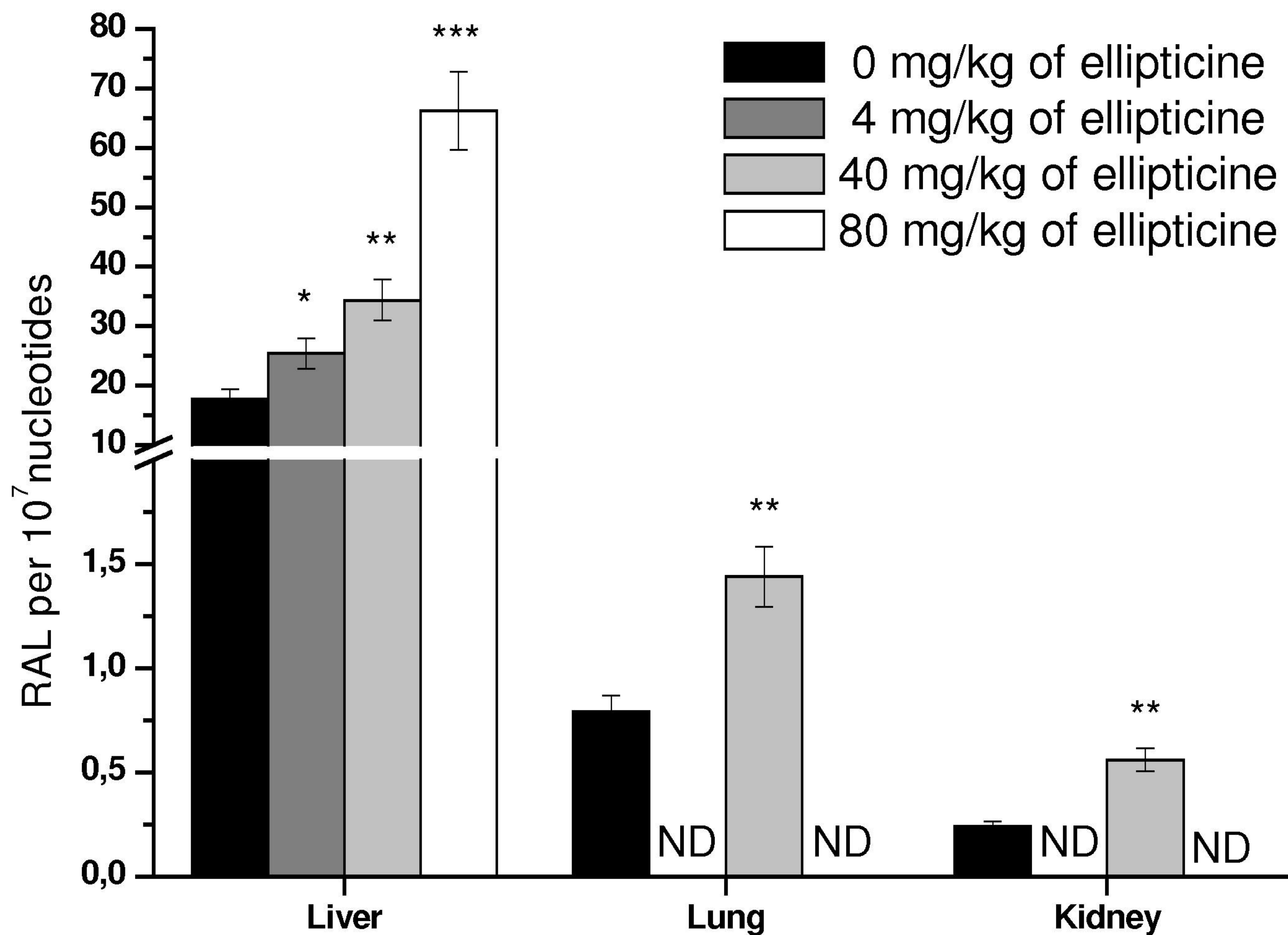

Figure 6

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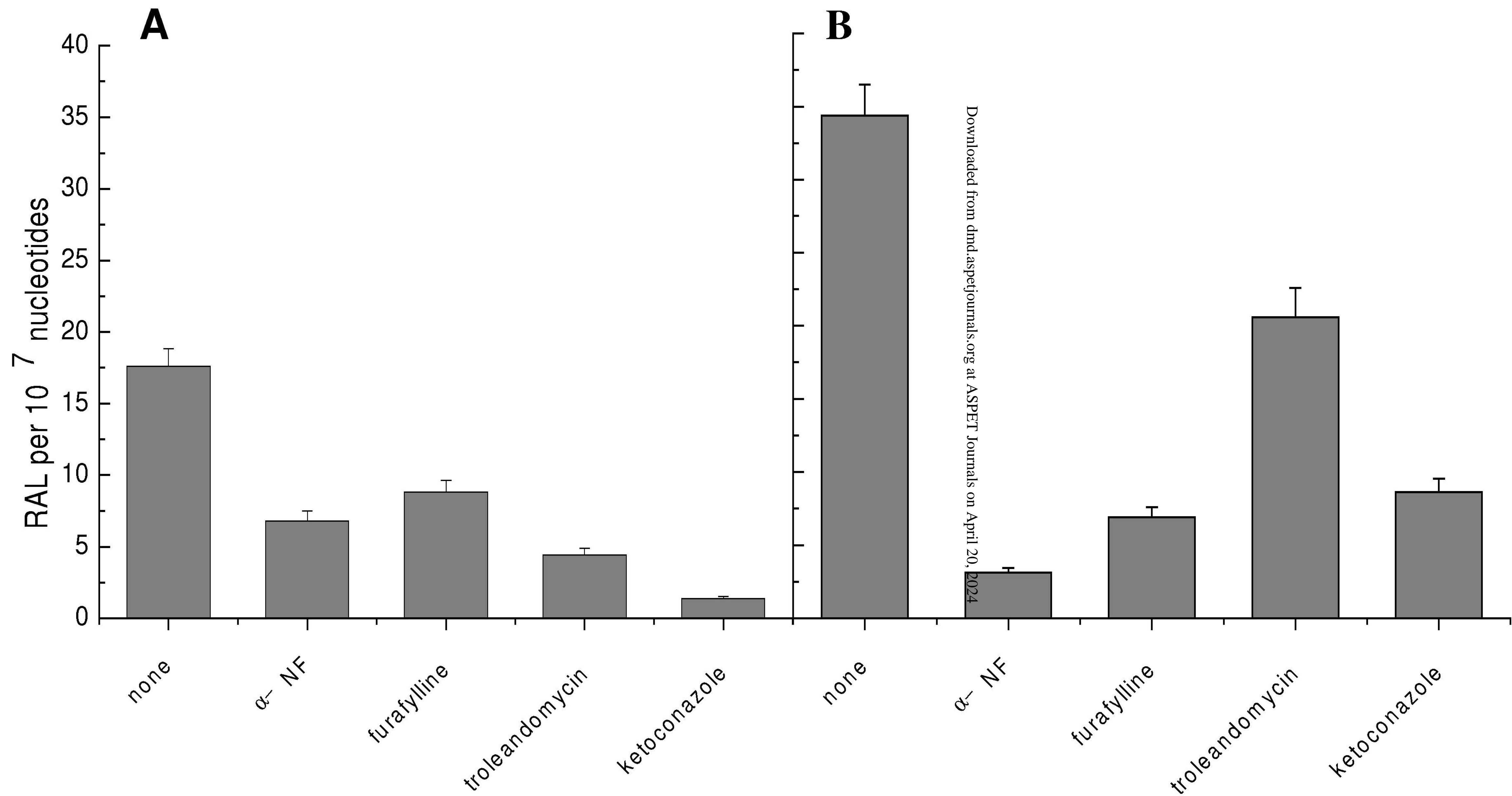

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Figure 7

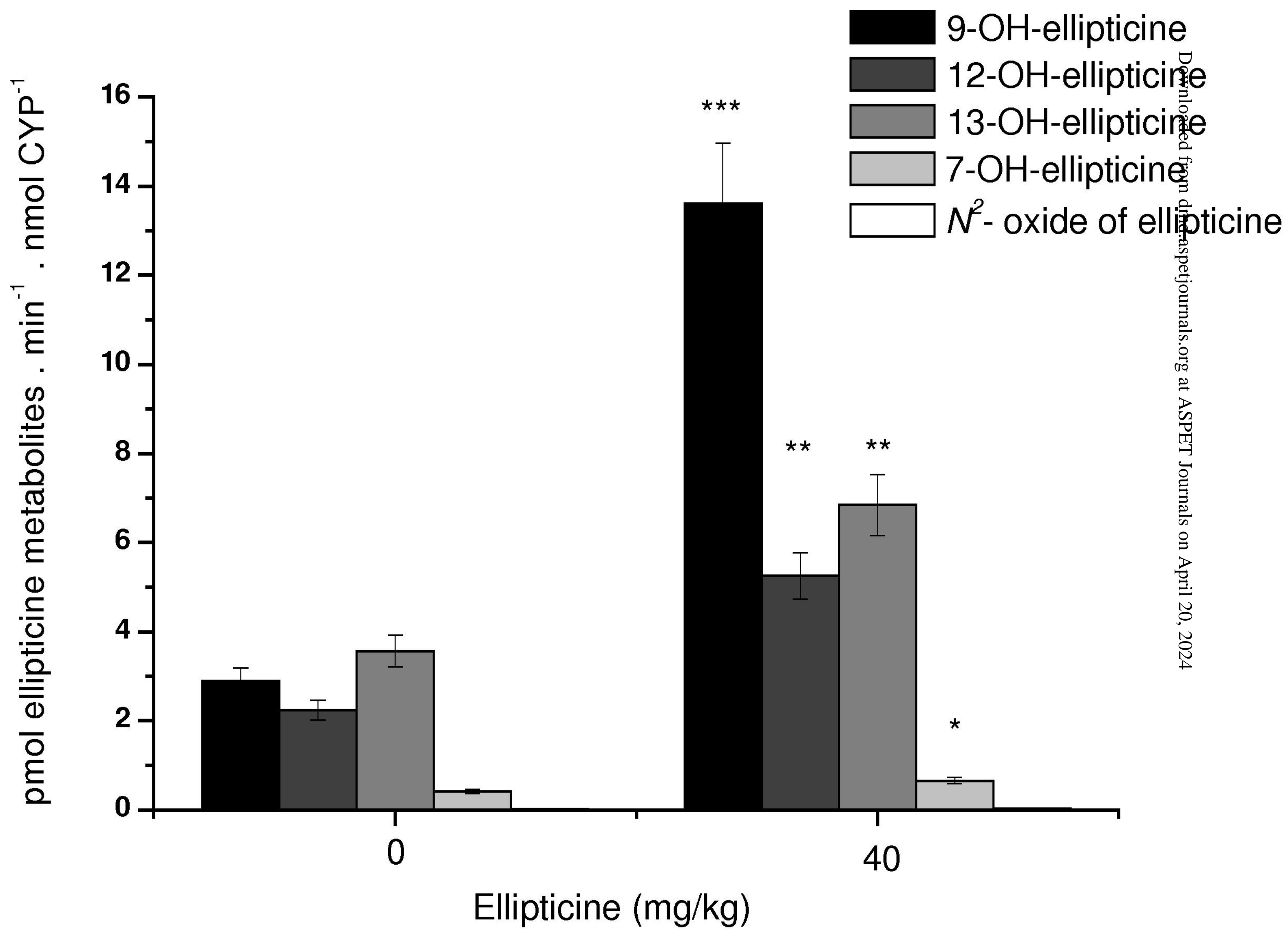

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Figure 8