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# Inhibition of CYP2B4 by the mechanism-based inhibitor 2-ethynylnaphthalene: Inhibitory potential of 2EN is dependent on the size of the substrate

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**Abstract**

2-Ethynylnaphthalene (2EN) is a mechanism-based inhibitor of CYP2B4 with two components to the inhibition, (1) enzyme inactivation, which requires covalent binding of the 2EN metabolite, and (2) reversible inhibition by 2EN itself. Both inhibitory components were examined using several different CYP2B4 substrates. Preincubation of CYP2B4 with 2EN led to a time-dependent inactivation of each of the CYP2B4-dependent activities examined; however, the ability of 2EN to reversibly inhibit CYP2B4 depended on the substrate employed, which is inconsistent with classical inhibition patterns. The degree 2EN's reversible inhibition was shown not to correlate with the substrate affinity for the active site, but with parameters related to the molecular size of the substrate. The results are consistent with 2EN and the smaller substrates simultaneously fitting in the CYP2B4 active site, leading to very little inhibition. Larger substrates exhibited greater degrees of inhibition because of their inability to co-bind with inhibitor in the active site.


*Keywords:* Cytochrome P450; CYP2B4; Mechanism-based inhibition; Enzyme kinetics; 2-Ethynylnaphthalene

Cytochrome P450 enzymes play a major role in oxidizing an extensive array of compounds including drugs, pesticides, carcinogens, steroids, and fatty acids. The identification of specific inhibitors and substrates for several of the P450 families has aided in the characterization of relative enzyme levels in microsomal preparations and has been useful as probes of the structure of the active site of specific P450 enzymes. Cytochrome P450 inhibitors can be divided into three categories that differ in mechanism: (a) agents that bind reversibly; (b) agents that form quasi-irreversible complexes with the heme iron atom; and (c) agents that bind irreversibly to the protein or the prosthetic heme group, or that accelerate degradation of the prosthetic heme group [1]. The second and third agents fall into the category of mechanism-based (or suicide) inhibitors. Three mechanisms for the inactivation of P450 by the mechanism-based inhibitors are known: *N*-alkylation of the prosthetic heme group; destruction of the heme; and covalent modification of the apoprotein by the reactive intermediate. The mechanism-based inactivation of P450 enzymes involves metabolic activation by the enzyme itself and is characterized by a time-dependent decrease in enzymatic activities. Dilution or dialysis of the enzyme:inhibitor solution does not dissociate the EI complex and restore enzyme activity. Reversible inhibitors interact with the enzyme through noncovalent association/dissociation reactions, and do not require prior metabolism of the inhibitor.

Certain compounds containing the acetylenic functional group have been shown to act both as reversible inhibitors

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and mechanism-based inactivators that alkylate the protein moiety of cytochrome P450 in microsomes and reconstituted systems [2–8]. Ethynes, such as 9-ethynylphenanthrene, are effective mechanism-based inhibitors of CYP2B-dependent reactions, whereas propynes like 1-(1-propynyl)-pyrene are more selective inhibitors of CYP1A enzymes [2,7]. 2-Ethynylnaphthalene (2EN)[1] has previously been shown to be a mechanism-based inactivator of P450, and the radiolabeled compound was found to irreversibly bind to the protein moiety of rat CYP2B1, rat CYP1A1 and CYP1A2, and rabbit CYP1A2 with a binding stoichiometry of approximately 0.5–1.3 nmol of adduct/nmol of P450 [4–6]. 2EN has also been reported to be an effective mechanism-based inhibitor of the 7-ethoxycoumarin *O*-deethylase activity of CYP2B4 with 0.83 mol of adduct bound per mole of P450 [2,3]. The catalysis of 2EN produces a highly reactive intermediate, 2-naphthylacetic acid, which covalently modifies the apoprotein and results in the inactivation.

Previous studies on mechanism-based inhibitors focused on the inactivation of CYP2B4 by 2EN using a substrate to monitor residual activity. In this study, we have investigated both the mechanism-based inactivation and the reversible inhibitory components of 2EN with seven different CYP2B4 substrates; *p*-nitroanisole (PNA), 7-ethoxycoumarin (7-EC), 7-ethoxy-4-trifluoromethylcoumarin (7-EFC), benzphetamine (BZP), 7-pentoxyresorufin (7-PR), testosterone (TS), and 7-benzyloxyresorufin (7-BR). Potential correlations between the reversible inhibitory component of 2EN and both the molecular characteristics and affinity of the substrate for CYP2B4 were examined. In this study, the reversible inhibition of CYP2B4 activity by 2EN was shown to be related to the size of the substrate, and not to the affinity of the substrate for the enzyme active site.

## Experimental procedures

### Materials

7-Ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC), 7-pentoxyresorufin (7-PR), 7-benzyloxyresorufin (7-BR), resorufin, dilauroylphosphatidylcholine (DLPC), nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, formaldehyde, and dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). Benzphetamine (BZP) was a gift from Upjohn (Kalamazoo, MI). 7-Ethoxy-4-trifluoromethylcoumarin (7-EFC) and 7-hydroxy-4-trifluoromethylcoumarin (7-HFC) were obtained from Molecular Probes (Eugene, OR). *p*-Nitroanisole (PNA) was provided by Acros Organics (Belgium). Testosterone (TS) and its metabolites were from Steraloids Inc. (Newport, RI). 2-Ethynylnaphthalene (2EN) was synthesized as described [5,6].

### Enzymes

Cytochrome P450 2B4 (CYP2B4) was expressed in *Escherichia coli* C41 and purified according to standard procedures [9]. NADPH–cytochrome P450 reductase was purified from phenobarbital-treated rabbits as described previously [10]. Recombinant rabbit NADPH–cytochrome P450 reductase (plasmid: pSC-CPR, provided by Dr. Lucy Waskell (University of Michigan); constructed from plasmid pCWori-rabbit reductase and plasmid pOR263-rat reductase, utilizing a T7 promoter) was expressed in *E. coli* C41, solubilized and purified according to a modification of previously described methods [11,12]. Both preparations of NADPH–cytochrome P450 reductase showed similar enzyme activity.

### Inhibition experiments: incubation with 2EN

For preparation of the reconstituted systems, 0.1 μM CYP2B4, 0.05 μM rabbit cytochrome P450 reductase, and 16 μM (dilauroylphosphatidylcholine) DLPC, were preincubated at room temperature for 2 h prior to dilution with the other assay components as described previously [12]. The reconstituted systems were then incubated at 37 °C with the mechanism-based inhibitor 2EN (1 μM), and an NADPH-regenerating system containing 0.5 mM NADPH, 5 mM glucose-6-phosphate, 2 U/ml glucose-6-phosphate dehydrogenase, and 10 mM $MgCl_2$ in 100 mM potassium phosphate buffer, pH 7.25 (*2EN reaction mixture*) for times ranging from 0 to 10 min. Control incubations contained an equivalent volume of DMSO in place of 2EN. For most experiments the reaction volume was 2 ml. 2EN was added from a stock solution in DMSO in all the experiments of this study. The reaction was initiated by the addition of NADPH. Following the incubation with 2EN, residual monooxygenase activity for numerous substrates was examined as described below.

### Measurement of monooxygenase activities

After preincubation with 2EN, metabolism of several different CYP2B4-dependent substrates was examined by addition of the substrate to the *2EN reaction mixture*. The substrates tested included 7-PR, 7-BR, 7-EC, 7-EFC, PNA, BZP, and TS. For 7-PR and 7-BR, the accumulation of resorufin was monitored fluorometrically at an excitation wavelength of 559 nm (for 7-PR) or 520 nm (for 7-BR) and an emission wavelength of 585 nm. Resorufin standards were used to determine the level of product formation [8,13]. Substrate concentrations ranged from 0.25 to 2.0 μM for 7-PR and 0.05 to 1.5 μM for 7BR.

For 7-EC and 7-EFC deethylation, the final concentrations ranged from 0.05 to 1 mM for 7-EC, and 0.005 to 0.1 mM for 7-EFC. The fluorescent products, 7-HC and 7-HFC, were monitored over a 3-min time course with excitation/emission wavelength of 368/456 nm for 7-HC or 410/510 nm for 7-HFC [14,15].

For PNA demethylation, formation of the product *p*-nitrophenol from substrate (from 0.005 to 0.5 mM) was monitored over a 5-min time trace by measuring the absorbance change at 405 nm. The amount of *p*-nitrophenol formed was calculated using an extinction coefficient of 12.87 $mM^{-1}$ $cm^{-1}$ at 405 nm [16].

A fluorometric method was used to determine BZP *N*-demethylation activity [17,18]. BZP (0.05–1 mM) was added to the *2EN reaction mixture* and incubated at 37 °C for 10 min. The reaction was terminated with 0.11 volumes of a mixture of 25% $ZnSO_4$ (w/v) and 0.5 M semicarbazide (10:1). The samples were neutralized with 0.1 volumes of saturated $Ba(OH)_2$ and placed on ice for 10 min and then centrifuged at 14,000*g* for 10 min. An aliquot (0.5 ml) of supernatant was mixed with 0.5 ml of Nash reagent [0.3% (v/v) acetic acid, 0.2% (v/v) acetylacetone, 15.4% (w/v) ammonium acetate]. The formaldehyde product was measured fluorometrically (excitation wavelength 410 nm, emission wavelength 510 nm) and was quantified using a standard curve.

[1] *Abbreviations used:* 2EN, 2-ethynylnaphthalene; PNA, *p*-nitroanisole; 7-EC, 7-ethoxycoumarin; 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin; BZP, benzphetamine; 7-PR, 7-pentoxyresorufin; 7-BR, 7-benzyloxyresorufin; TS, testosterone; DLPC, dilauroylphosphatidylcholine; NADPH, nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt; DMSO, dimethylsulfoxide; 7-HFC, 7-hydroxy-4-trifluoromethylcoumarin; 7-HC, 7-hydroxycoumarin; $K_{inact}$, the dissociation constant for the irreversible inactivation of the enzyme; $K_i^{rev}$, the dissociation constant for reversible inhibition; CYP2B4, cytochrome P450 2B4; CYP1A2, cytochrome P450 1A2; CYP1A1, cytochrome P450 1A1.