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5-ETHYNYL-2(1*H*)-PYRIMIDINONE: ALDEHYDE OXIDASE-ACTIVATION TO 5-ETHYNYLURACIL, A MECHANISM-BASED INACTIVATOR OF DIHYDROPYRIMIDINE DEHYDROGENASE

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Abstract—5-Ethynyluracil is a potent mechanism-based inactivator of dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) *in vitro* (Porter *et al.*, *J Biol Chem* **267**: 5236–5242, 1992) and *in vivo* (Spector *et al.*, *Biochem Pharmacol*, **46**: 2243–2248, 1993. 5-Ethynyl-2(1*H*)-pyrimidinone was rapidly oxidized to 5-ethynyluracil by aldehyde oxidase. The substrate efficiency (k_{cat}/K_m) was 60-fold greater than that for *N*-methylnicotinamide. In contrast, xanthine oxidase oxidized 5-ethynyl-2(1*H*)-pyrimidinone to 5-ethynyluracil with a substrate efficiency that was only 0.02% that of xanthine. Because 5-ethynyl-2(1*H*)-pyrimidinone did not itself inactivate purified DPD *in vitro* and aldehyde oxidase is predominately found in liver, we hypothesized that 5-ethynyl-2(1*H*)-pyrimidinone could be a liver-specific inactivator of DPD. We found that 5-ethynyl-2(1*H*)-pyrimidinone administered orally to rats at 2 μg/kg inactivated DPD in all tissues studied. Although 5-ethynyl-2(1*H*)-pyrimidinone produced slightly less inactivation than 5-ethynyluracil, the two compounds showed fairly similar patterns of inactivation of DPD in these tissues. At doses of 20 μg/kg, however, 5-ethynyl-2-pyrimidinone and 5-ethynyluracil produced equivalent inactivation of DPD. Thus, 5-ethynyl-2(1*H*)-pyrimidinone appeared to be an efficient, but not highly liver-selective prodrug of 5-ethynyluracil.

Key words: 5-ethynyl-2(1*H*)-pyrimidinone; 5-ethynyluracil; dihydropyrimidine dehydrogenase; aldehyde oxidase; xanthine oxidase

Dihydropyrimidine dehydrogenase, the initial enzyme in the catabolism of thymine and uracil, is responsible for the degradation of over 80% of 5-FU§ administered to cancer patients [1]. Because the activity of this enzyme varies temporally in an individual, the plasma levels of constantly infused 5-FU vary considerably (3-fold) over a 24-hr period [1]. The therapeutic efficacy and toxicity of 5-FU may be dependent on the activity of DPD [2]. Thus, 5-bromovinyluracil, which inactivates DPD in tissue extracts, increases the antitumor activity of 5-FU in mice inoculated with leukemia cells [2]. These results prompted us to search for more efficient inactivators of DPD. We found that 5-ethynyluracil is a potent mechanism-based inactivator of purified DPD [3]. 5-Ethynyluracil initially forms a reversible complex ($K_d = 1.6\ \mu M$) with DPD that subsequently reacts ($20\ min^{-1}$) to form covalently modified enzyme [3]. Several advantages have been demonstrated for treating tumor-bearing animals with 5-ethynyluracil prior to dosing with 5-FU. First, 5-ethynyluracil increases the therapeutic index as well as the efficacy of 5-FU [4, 5]. Second, 5-ethynyluracil improves the bioavailability of 5-FU [6]. Finally, 5-ethynyluracil decreases the variability and increases the half-life of 5-FU in plasma of rats dosed orally with 5-FU [6].

Prodrugs are usually developed to increase the oral bioavailability of a drug. For example, 6-deoxyacyclovir and 6-aminoacyclovir have been shown to be prodrugs of the antiherpetic agent acyclovir [7, 8]. In the former case, 6-deoxyacyclovir is oxidized by xanthine oxidase to acyclovir [8]. The average plasma concentration of acylovir from a 200 mg dose of 6-deoxyacyclovir is 6-fold larger than that from the same dose of acyclovir [8]. Because the bioavailability of 5-ethynyluracil is greater than 60%‖, efforts to develop a prodrug that only increases the bioavailability of 5-ethynyluracil are not warranted. However, the prodrug strategy can also prove advantageous when the prodrug is only metabolized to the active drug in a targeted organ and could thereby increase the specificity of the drug. For example, aldehyde oxidase oxidizes 5-substituted-2-pyrimidinones to their corresponding uracil derivatives [9–12]. Specifically, 5-fluoro-2-pyrimidinone is oxidized to 5-FU by aldehyde oxidase [10]. Because aldehyde oxidase activity is highest in liver [11], 5-FU might be generated selectively from 5-fluoro-2-pyrimidinone in the liver. Similarly, the antiherpetic agent 5-iodo-2′-deoxyuridine can be generated by the enzymatic oxidation of 5-iodo-2-pyrimidinone nucleoside [9]. If 5-ethynyl-2(1*H*)-pyrimidinone (II), the analogous precursor to 5-ethynyluracil (I), were oxidized by

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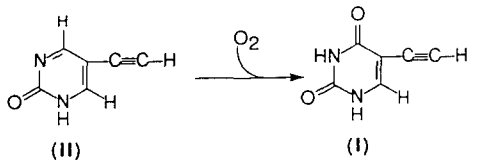
§ Abbreviations: DPD, dihydropyrimidine dehydrogenase; and 5-FU, 5-fluorouracil.

‖ Nelson DJ and Frick LW, unpublished data. Cited with permission.

aldehyde oxidase (Scheme I), DPD might be selectively inhibited in the liver.



Scheme 1

In the present report, we have demonstrated that 5-ethynyl-2(1*H*)-pyrimidinone was oxidized efficiently by purified aldehyde oxidase and inefficiently by xanthine oxidase. However, orally dosed 5-ethynyl-2-pyrimidinone and 5-ethynyluracil showed similar patterns of inactivation of DPD in a variety of tissues removed from treated rats. Consequently, 5-ethynyl-2(1*H*)-pyrimidinone appeared to be an efficient prodrug of 5-ethynyluracil, but did not have a significant organ specificity advantage.

MATERIALS AND METHODS

Materials. Frozen rabbit liver was obtained from Pel-Freez (Rogers, AR). *N*-Methylnicotinamide, catalase (C-100), and xanthine were from the Sigma Chemical Co. (St. Louis, MO). Whatman DE-52 was from Bodman (Media, PA), P-6 resin from Bio-Rad (Richmond, CA), 5-ethynyluracil from the Burroughs Wellcome Co. (Research Triangle Park, NC), and xanthine oxidase from Boehringer Mannheim (Danbury, CT). Dihydropyrimidine dehydrogenase was purified from bovine liver and assayed spectrophotometrically as described previously [13].

Synthesis of 5-ethynyl-2(1H)-pyrimidinone. In a modification of the procedure of Bardos and coworkers [14], 5-iodo-2(1*H*)-pyrimidinone [15] (17.2 g 77.5 mmol) was added to hexamethyldisilazane (150 mL) in an oven-dried 500-mL three-neck round bottom flask equipped with two rubber septa and a condenser with a nitrogen inlet. Chlorotrimethylsilane (3.5 mL) was added, and the suspension was heated at reflux for 1 hr. After cooling, the red–brown mixture was quickly filtered through a Celite pad and washed with anhydrous toluene (30 mL). The filtrate was transferred to an oven-dried 250-mL round bottom flask and concentrated *in vacuo* to a dark brown oil. The oil was degassed and diluted with triethylamine (60 mL freshly distilled from potassium hydroxide) and anhydrous dimethylformamide (30 mL). Trimethylsilylacetylene (13.4 mL, 93 mmol) was added portionwise followed by commercial 98% bis(triphenylphosphine)palladium(II) chloride (0.55 g, 0.77 mmol) and copper iodide (0.37 g, 1.9 mmol). The reaction, which was stirred for 17 hr at room temperature, became biphasic with a dark lower layer and a pale yellow upper layer. Removal of solvent *in vacuo* was followed by the addition of tetrahydrofuran (200 mL) and filtration through a Celite pad. The pad was washed with tetrahydrofuran (200 mL), and the combined filtrates were concentrated *in vacuo* to a dark oil. Methanol (75 mL) and ethyl acetate (30 mL) were added and the mixture was swirled vigorously for 15 min. The resulting beige precipitate was filtered, washed with chloroform (50 mL), and air-dried for 30 min. Further drying under high vacuum for a 2-hr period provided 5-(2-trimethylsilyl)-ethynyl-2(1*H*)-pyrimidinone (10.8 g, 56.3 mmol, 73%) as a pale beige powder. ^{1}H-NMR (d_6-DMSO): δ 12.40 (bs, NH), 8.44 (bs, H4 and H6), 0.22 (s, $SiMe_3$). Anal. Calcd. for $C_9H_{12}N_2OSi$ (1.00 CH_3OH): C, 53.54; H, 7.19; N, 12.49. Found: C, 53.52; H, 7.12; N, 12.67.

To a suspension of 5-(2-trimethylsilyl)-ethynyl-2(1*H*)-pyrimidinone (0.75 g, 3.9 mmol) in anhydrous tetrahydrofuran (20 mL) in a 100-mL three-neck round bottom flask equipped with two rubber septa and a nitrogen inlet was added a 1.0 M solution of tetrabutylammonium fluoride in tetrahydrofuran (4.7 mL, 4.7 mmol). The solution was stirred under nitrogen for 17 hr and then concentrated *in vacuo* to a brown solid. Ethyl acetate (50 mL) and silica gel (5 g) were added, and the slurry was concentrated to a beige powder that was preloaded atop a column of silica gel. Flash chromatography with chloroform : methanol (98 : 2 to 90 : 10) in increments of increasing polarity provided 5-ethynyl-2(1*H*)-pyrimidinone as a pale yellow powder (0.34 g, 2.8 mmol, 73%). ^{1}H-NMR (d_6-DMSO): δ 12.37 (bs, NH), 8.45 (s, H4 and H6), 4.27 (s, H–C≡C). Anal. Calcd. for $C_6H_4N_2O$: C, 60.00; H, 3.36; N, 23.32. Found: C, 59.80; H, 3.49; N, 23.06. This material was estimated by HPLC to be of greater than 98% purity based upon total absorbance at 260 nm. For enzymatic studies, small quantities of 5-ethynyl-2(1*H*)-pyrimidinone were purified further by HPLC to remove trace contaminants. The HPLC system used to identify 5-ethynyluracil as a product of the enzymatic reactions was adapted for this purification step (see below).

Identification of 5-ethynyluracil as the oxidation product of 5-ethynyl-2(1H)-pyrimidinone. 5-Ethynyluracil was demonstrated to be the product of enzymatic oxidation of 5-ethynyl-2(1*H*)-pyrimidinone by comparison of the retention time and the ultraviolet spectrum of the product eluting from a reverse-phase HPLC column with those of authentic 5-ethynyluracil. Samples were deproteinated prior to analysis for 5-ethynyluracil by ultrafiltration with a Centrifree-30 (Amicon, Beverly, MA). 5-Ethynyluracil and 5-ethynyl-2(1*H*)-pyrimidinone were separated on a LiChroCart 250-4 100 RP-18 reverse-phase column (Catalogue No. 50838, E. Merck, Germany) that was developed isocratically in 10 mM ammonium phosphate at pH 6.8. The absorbance was monitored at 210 and 260 nm. The spectra of the products were recorded with a Kontron Diode Array Detector DAD 440 (Everrett, MA). A 100-μL sample was applied to the column, and the column was developed with a flow rate of 1 mL/min. Under these conditions 5-ethynyluracil had a retention time of 12.0 min and 5-ethynyl-2(1*H*)-pyrimidinone had a retention time of 13.6 min. The amount of 5-ethynyluracil and 5-ethynyl-2(1*H*)-pyrimidinone in the sample was determined by comparison of the area under the peak in the 260 nm chromatogram with that for standard solutions with

known amounts of 5-ethynyluracil or 5-ethynyl-2(1*H*)-pyrimidinone.

Preparation of xanthine oxidase and aldehyde oxidase. Xanthine oxidase was received from the manufacturer as an ammonium sulfate suspension. In most cases, ammonium sulfate was removed by disc dialysis at 5° against 0.05 M potassium phosphate at pH 7.0. When the enzyme was used without removal of ammonium sulfate by dialysis, similar results were observed. Xanthine oxidase was assayed spectrophotometrically at 292 nm ($\Delta\varepsilon_{292}$ = 9.75 mM^{-1} cm^{-1} [16]) with 10 μM xanthine. One unit of xanthine oxidase catalyzed the oxidation of 1 μmol xanthine/min.

Aldehyde oxidase was partially purified from rabbit liver [17–19]. Rabbit liver (200 g) was homogenized in 200 mL of 0.05 M Tris–HCl (pH 8.0) and 0.5 mM EDTA (buffer A) for 3 min at high speed with a food blender. The mixture was diluted to 1 L with buffer A and centrifuged at 10,000 *g* for 20 min. An equal volume of a 50% suspension of DE-52 resin (900 mL) was added to the supernatant at 5°. The mixture was stirred for 20 min. The resin was collected by filtration on a scintered glass funnel and washed with 1000 mL of buffer A and 1500 mL of buffer A supplemented with 0.1 M KCl. Aldehyde oxidase was eluted from the resin with 1000 mL of buffer A supplemented with 0.2 M KCl. The effluent was made 35% in ammonium sulfate (209 g/L) and stirred for 20 min. The precipitated protein was removed by centrifugation for 20 min at 10,000 *g*. Aldehyde oxidase was precipitated with 45% (63 g/L) ammonium sulfate. After 20 min, the precipitated protein was collected by centrifugation. The pellet was dissolved in 8 mL of buffer A and heated at 50° for 2 min. The denatured protein was removed by centrifugation, and residual ammonium sulfate was removed on a column of P-6 resin equilibrated in buffer A. The activity of aldehyde oxidase was determined with 0.15 mM *N*-methylnicotinamide in 0.05 M potassium phosphate and 0.5 mM EDTA at pH 7.8 and 25°. Product formation was monitored by the increase in absorbance at 300 nm ($\Delta\varepsilon_{300}$ = 4.2 mM^{-1} cm^{-1} [19]). One unit of aldehyde oxidase catalyzed the oxidation of 1 μmol *N*-methylnicotinamide/min. Protein was determined by the Coomassie Blue-binding method using the protein reagent supplied by Bio-Rad with bovine serum albumin as a protein standard [20]. The purification procedure resulted in a 140-fold increase in specific activity of the enzyme to 0.34 μmol/min/mg in 15% yield. This preparation of aldehyde oxidase had no detectable activity with xanthine.

In vivo *inactivation of DPD by 5-ethynyluracil and 5-ethynyl-2(1H)-pyrimidinone.* Rats (2 per group) were dosed orally with either 0, 2, or 20 μg/kg 5-ethynyluracil or 5-ethynyl-2(1*H*)-pyrimidinone. Samples of intestine, lung, spleen, and brain were removed after 1 hr. Tissue from animals receiving no drug were treated separately to determine the individual variability. Pairs of organs from animals receiving 5-ethynyluracil or 5-ethynyl-2(1*H*)-pyrimidinone were combined. Tissue extracts were prepared and DPD was assayed in tissue extracts by determining the amount of $^{14}CO_2$ enzymatically released from [2-^{14}C]uracil[2] [21].

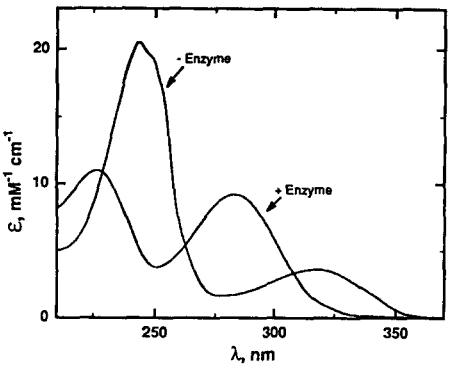


Fig. 1. Spectral changes associated with the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone by aldehyde oxidase. The spectrum of 53 μM 5-ethynyl-2(1*H*)-pyrimidinone was recorded in 0.05 M potassium phosphate at pH 7.0 and 37° prior to the addition of enzyme (−enzyme) and 5 min after the addition of 0.03 U of aldehyde oxidase (+enzyme). The spectrum of the product was identical to 5-ethynyluracil.

Data analysis. The Michaelis–Menten equation was fitted to initial velocity data by a nonlinear least squares fitting routine to yield estimates for K_m and V_{max} values. The integrated rate equation was fitted to the time–course for oxidation of xanthine by xanthine oxidase to yield estimates for K_m and V_{max} values [16]. Equation 1 was fitted by a nonlinear least squares fitting routine to the time–course for enzyme inactivation.

$$[\text{Product}] = A \cdot (1 - \exp(-b \cdot t)) \quad (1)$$

where A is the amplitude and b is the first-order rate constant for the reaction. Nonlinear fitting routines were performed with SigmaPlot (Corte Madera, CA).

RESULTS

Interaction of DPD with 5-ethynyl-2(1H)-pyrimidinone. DPD did not catalyze (<1% the rate of uracil reduction) the reduction of 5-ethynyl-2(1*H*)-pyrimidinone (25 μM) by NADPH (100 μM). Because the apparent K_m of DPD for uracil [1.8 ± 0.6 μM (N = 2)] in the presence of 200 μM NADPH was unaffected by 100 μM 5-ethynyl-2(1*H*)-pyrimidinone [1.8 ± 0.2 μM (N = 2)], 5-ethynyl-2(1*H*)-pyrimidinone was an inefficient inhibitor of DPD. Finally, DPD was not inactivated (<5%) after a 15-min incubation with 100 μM 5-ethynyl-2(1*H*)-pyrimidinone at 25°, whereas incubation of the enzyme with 100 μM 5-ethynyluracil resulted in >95% inactivation.

Oxidation of 5-ethynyl-2(1H)-pyrimidinone by aldehyde oxidase. Because the absorbance spectra of 5-ethynyluracil and 5-ethynyl-2(1*H*)-pyrimidinone were very different, the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone to 5-ethynyluracil was monitored spectrophotometrically. Based on these spectral differences, aldehyde oxidase (0.03 U/mL) completely oxidized 53 μM 5-ethynyl-2(1*H*)-pyrimidinone to 5-ethynyluracil within 4 min in air-saturated 0.05 M potassium phosphate at pH 7.0 and 37° (Fig. 1). Analysis of the reaction mixture by

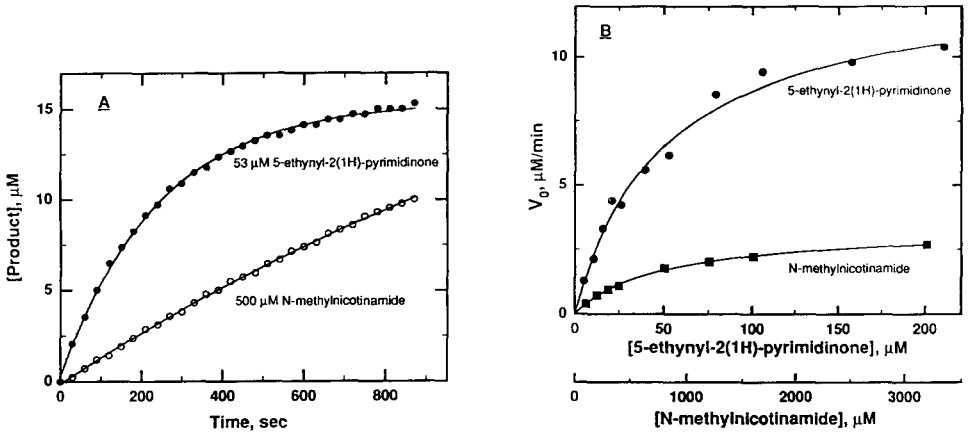


Fig. 2. Kinetics for the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone and *N*-methylnicotinamide by aldehyde oxidase. (A) Time–course for oxidation of *N*-methylnicotinamide and 5-ethynyl-2(1*H*)-pyrimidinone by aldehyde oxidase at pH 7.0 and 37°. The time–courses for the oxidation of 53 μM 5-ethynyl-2(1*H*)-pyrimidinone (K_m = 50 μM) or 500 μM *N*-methylnicotinamide (K_m = 750 μM) were monitored after the addition of 0.003 U/mL of aldehyde oxidase. The solid lines were calculated by equation 1 for a burst in *N*-methylnicotinamide oxidization equal to 28 ± 2 μM and a first-order rate constant for inactivation of $(5.2 \pm 0.4)10^{-4}$ sec^{-1}. The analogous values for the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone were 15.2 ± 0.1 μM and $(4.11 \pm 0.07)10^{-3}$ sec^{-1}. (B) Dependence of the initial velocity of 5-ethynyl-2(1*H*)-pyrimidinone oxidation on the concentration of 5-ethynyl-2(1*H*)-pyrimidinone. The solid lines were calculated for the fit of the Michaelis–Menten equation to these data. The concentration of aldehyde oxidase was 0.003 U/mL.

reverse-phase HPLC indicated a single species that had the retention time (12.0 min) and spectral properties of 5-ethynyluracil (Fig. 1). 5-Ethynyl-2(1*H*)-pyrimidinone, which had a retention time of 13.6 min, was not detected in the aldehyde oxidase-treated sample. Since our preparation of aldehyde oxidase did not catalyze the oxidation of xanthine, the 5-ethynyl-2(1*H*)-pyrimidinone-oxidizing activity was not the result of contaminating xanthine oxidase (see below). Furthermore, the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone by this enzyme preparation was inhibited 50% by 2 mM dithiothreitol, 35% by 0.5 mM zinc chloride and over 95% by 2 μM cupric acetate, but the reaction was stimulated 1.7-fold by 100 μM potassium ferricyanide. The effects of these reagents on the oxidation of 5-ethynyl-2-pyrimidinone by this enzyme preparation were characteristic of aldehyde oxidase [17–19].

The kinetics for the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone and *N*-methylnicotinamide by aldehyde oxidase in 0.05 M potassium phosphate (pH 7.0) at 37° were monitored spectrophotometrically at 325 nm ($\Delta\varepsilon_{325} = -2.4$ mM^{-1} cm^{-1}) and 300 nm ($\Delta\varepsilon_{300} = 4.2$ mM^{-1} cm^{-1}), respectively (Fig. 2A). The steady-state kinetic parameters for the oxidation of these substrates were determined from initial velocity data. The K_m of aldehyde oxidase for *N*-methylnicotinamide was 750 ± 40 μM, and the relative maximal velocity was defined to be 1. The K_m of aldehyde oxidase for 5-ethynyl-2(1*H*)-pyrimidinone was 50 ± 5 μM, and the relative maximal velocity was 3.9 (Fig. 2B). Based on the ratio V_{max}/K_m, 5-ethynyl-2(1*H*)-pyrimidinone was oxidized 60-fold more efficiently than *N*-methylnicotinamide. 5-Ethynyluracil (50 μM) did not inhibit detectably the oxidation of 500 μM *N*-methylnicotinamide by aldehyde oxidase.

Aldehyde oxidase was progressively inhibited during the oxidation reaction. Thus, aldehyde oxidase (0.003 U/mL) partially oxidized 53 μM 5-ethynyl-2(1*H*)-pyrimidinone (30%) before the reaction ceased (Fig. 2A). Because addition of an equal amount of enzyme resulted in a similar burst in product formation (data not shown), the oxidation stopped because the enzyme was inactivated and not because the enzyme was inhibited by product. The first-order rate constant for inactivation was $(4.11 \pm 0.07)10^{-3}$ sec^{-1}. The time–course for oxidation of 500 μM *N*-methylnicotinamide was similar to that for the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone, indicating again that aldehyde oxidase was inactivated during catalysis (Fig. 2A). Equation 1 was fitted to these data to yield a burst in product formation of 28 μM and a first-order inactivation rate constant of $(5.2 \pm 0.4)10^{-4}$ sec^{-1}. The apparent constant for inactivation of aldehyde oxidase during the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone was decreased 50% in the presence of 10 μg/mL catalase and 10 μg/mL superoxide dismutase. The rate of inactivation was not affected by 1 mM EDTA.

Oxidation of 5-ethynyl-2(1H)-pyrimidinone by xanthine oxidase. Xanthine oxidase caused changes in the spectrum of 5-ethynyl-2(1*H*)-pyrimidinone that were similar to those observed with aldehyde oxidase. Thus, 0.2 U/mL xanthine oxidase catalyzed the slow oxidation of 5-ethynyl-2(1*H*)-pyrimidinone ($t_{1/2}$ > 100 min) in air-saturated 0.05 M potassium phosphate (pH 7.0) at 37°. The spectral changes associated with this reaction had isosbestic points at 229, 262 and 308 nm. The difference spectra between

the substrate and the product had minima at 326 and 248 nm and a maximum at 282 nm. The difference extinction coefficient at 326 nm was $-2.4\ mM^{-1}\ cm^{-1}$. These spectral properties were identical to those for the difference spectrum between 5-ethynyluracil and 5-ethynyl-2(1*H*)-pyrimidinone. The product of this reaction was confirmed to be 5-ethynyluracil by HPLC. The deproteinated reaction mixture yielded two components on reverse-phase HPLC, a species with a retention time of 13.6 min, which corresponded to 5-ethynyl-2(1*H*)-pyrimidinone, and a new species with a retention time (12.0 min) and spectral properties of 5-ethynyluracil. After a 120-min incubation, approximately 60% of 5-ethynyl-2(1*H*)-pyrimidinone was oxidized to 5-ethynyluracil. Xanthine oxidase also lost catalytic activity during the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone. Thus, the reaction of 200 μM 5-ethynyl-2(1*H*)-pyrimidinone with 0.2 U/mL xanthine oxidase for 2 hr at 37° resulted in the loss of 44% of the catalytic activity. The inactivation process was not prevented by catalase (10 μg/mL), superoxide dismutase (10 μg/mL) or EDTA (1.0 mM). Because the inactivation reaction was only significant after prolonged incubation of the enzyme with 5-ethynyl-2(1*H*)-pyrimidinone, it was not investigated further.

Initial velocity of the oxidation of 5-ethynyl-2-(1*H*)-pyrimidinone by xanthine oxidase was monitored spectrophotometrically at 282 nm ($\Delta\varepsilon_{282} = 7.6\ mM^{-1}\ cm^{-1}$). The K_m for 5-ethynyl-2(1*H*)-pyrimidinone was 310 ± 50 μM and the V_{max} of 5-ethynyl-2(1*H*)-pyrimidinone relative to 1.0 for xanthine was 0.028. The K_m value for the oxidation of xanthine by xanthine oxidase under these experimental conditions was estimated to be 1.7 ± 0.2 μM from the time–course for oxidation of 6 μM xanthine and the integrated rate equation. In an independent experiment, the K_m of xanthine oxidase for xanthine was increased marginally by 115 μM 5-ethynyluracil from a value of 2.1 ± 0.02 μM to a value of 3.1 ± 0.2 μM.

Because the rate of oxidation of 5-ethynyl-2(1*H*)-pyrimidinone by this preparation of xanthine oxidase was less than 3% the rate of oxidation of xanthine, it was possible that 5-ethynyl-2(1*H*)-pyrimidinone was being oxidized by a contaminating enzyme in the xanthine oxidase preparation. To eliminate this possibility, the effect of oxypurinol, a specific inhibitor of xanthine oxidase, on the rate of oxidation of 5-ethynyl-2(1*H*)-pyrimidinone was examined. The K_i of bovine xanthine oxidase for oxypurinol is 100 nM [16]. However, the binding of this inhibitor to xanthine oxidase is a two-step process. The inhibitor forms an initial complex with a K_d of 7.2 μM that subsequently isomerizes to a much tighter complex if the enzyme is in the reduced state. Thus, 13 μM oxypurinol reduced the initial rate of oxidation of 30 μM 5-ethynyl-2(1*H*)-pyrimidinone by 68%. The rate of oxidation was relatively constant over a 30-min reaction. In contrast, when the enzyme preparation was treated with 55 μM xanthine, a substrate that reduces the enzyme, and 13 μM oxypurinol for 5 min prior to addition of 30 μM 5-ethynyl-2(1*H*)-pyrimidinone, the tight complex formed so that the slow oxidation of 5-ethynyl-2(1*H*)-pyrimidinone was inhibited completely. These results indicated that 5-ethynyl-2(1*H*)-pyrimidinone was oxidized by xanthine oxidase and not by a contaminating protein.

Comparison of the activity of DPD in selected tissues of rats treated with 5-ethynyluracil or 5-ethynyl-2(1H)-pyrimidinone. The extent of inactivation of DPD in tissues from rats treated with 5-ethynyluracil or 5-ethynyl-2(1*H*)-pyrimidinone was determined as described in Materials and Methods. Treatment of rats with 20 μg/kg 5-ethynyluracil or 5-ethynyl-2(1*H*)-pyrimidinone resulted in similar inactivation of DPD in liver, intestine, lung, spleen and brain. Thus, 5-ethynyl-2(1*H*)-pyrimidinone was as effective as 5-ethynyluracil as an inactivator of DPD at this dose. At the lower dose (2 μg/kg) of 5-ethynyluracil and 5-ethynyl-2(1*H*)-pyrimidinone, the ratios of the extent of inactivation by 5-ethynyluracil to that by 5-ethynyl-2(1*H*)-pyrimidinone, which is a measure of the selectivity of 5-ethynyl-2(1*H*)-pyrimidinone, differed by less than a factor of two for the tissues examined (Table 1).

DISCUSSION

Krenitsky *et al.* [8] have synthesized 6-deoxyacyclovir as an acyclovir prodrug that is activated oxidatively by xanthine oxidase. This prodrug was designed to increase the bioavailability of acyclovir. Liver aldehyde oxidase efficiently catalyzes the oxidation of 5-substituted-2-pyrimidinone precursors of pyrimidine and pyrimidine nucleosides to the respective pyrimidine or pyrimidine nucleoside [9, 10, 12]. For example, prodrugs for the antineoplastic agents, 5-FU and 5-iodo-2′-deoxyuridine, were designed from appropriate 5-substituted-2-pyrimidinone analogs. These analogs were efficiently converted to the active agents by liver aldehyde oxidase. The prodrugs of 5-iodo-2′-deoxyuridine and 5-FU were claimed to introduce additional selectivity resulting from their selective oxidation to their active form in the targeted organ [10]. Because of this additional selectivity, it has been proposed that prodrugs activated by aldehyde oxidase should be considered in "hepatotrophic drug design" [9, 10].

DPD, which is responsible for the degradation of over 80% of continuously infused 5-FU, probably modulates the therapeutic efficacy of 5-FU. Indeed, 5-ethynyluracil has been found to be a potent inactivator of DPD *in vivo* that enhances the therapeutic index of 5-FU [4, 5]. If the catabolites arising from the reduction of 5-FU are not the major cause of 5-FU toxicity, then DPD would be a primary mechanism for detoxification of 5-FU. Thus, greater selectivity of 5-FU might be achieved if DPD were inactivated only in the targeted organ. Accordingly, an inactivator of DPD that selectively inactivated the liver enzyme might increase the therapeutic index of 5-FU for liver cancer over the increase observed with 5-ethynyluracil alone. Based on the potency of 5-ethynyluracil as an *in vivo* inactivator of DPD [21] and the reported substrate specificity of aldehyde oxidase [9, 10, 12], 5-ethynyl-2(1*H*)-pyrimidinone was selected as a precursor for 5-ethynyluracil. Because Cheng and coworkers [10] had shown that liver aldehyde oxidase efficiently

Table 1. Comparison of the activity of DPD in selected tissues of rats 1 hr after oral treatment with 5-ethynyl-2(1*H*)-pyrimidinone or 5-ethynyluracil

Tissue	Specific activity of DPD* (pmol/min/mg)	% Inactivation at specified dose			
		2 μg/kg		20 μg/kg	
		5-Ethynyluracil	5-Ethynyl-2(1*H*)-pyrimidinone	5-Ethynyluracil	5-Ethynyl-2(1*H*)-pyrimidinone
Liver	400 ± 100	74 ± 2	60 ± 10	90 ± 5	92 ± 3
Intestine	300 ± 100	60 ± 8	30 ± 10	91 ± 1	88 ± 4
Lung	22 ± 3	44 ± 8	30 ± 7	80 ± 10	86 ± 3
Spleen	14 ± 5	50 ± 30	30 ± 40	90 ± 2	90 ± 4
Brain	10	12	18	60	63

* Specific activity of DPD in tissue homogenates from untreated rats. Values are means ± range of two experiments except for brain where only a single determination was made.

oxidizes 5-iodo-2-pyrimidinone and 5-ethynyl-2(1*H*)-pyrimidinone-2′-deoxyribose, we hypothesized that this enzyme would also oxidize 5-ethynyl-2(1*H*)-pyrimidinone, as outlined in Scheme 1. We found that aldehyde oxidase oxidized 5-ethynyl-2(1*H*)-pyrimidinone to 5-ethynyluracil approximately 60-fold more efficiently than *N*-methylnicotinamide, the standard substrate for this enzyme. In contrast, xanthine oxidase catalyzed this oxidation inefficiently with a rate that was only 0.02% of that for xanthine.

Aldehyde oxidase was slowly inactivated during the oxidation of 5-ethynyl-2(1*H*)-pyrimidinone. The inactivation process was not totally eliminated by catalase and superoxide dismutase. However, the enzyme was also inactivated by the reference substrate *N*-methylnicotinamide. Because the first-order rate constants for inactivation of aldehyde oxidase by *N*-methylnicotinamide and 5-ethynyl-2(1*H*)-pyrimidinone were similar after normalization to the velocity of product formation, the inactivation event was probably not a property of 5-ethynyl-2(1*H*)-pyrimidinone *per se* but was a property of the enzyme during turnover with most substrates.

We had hypothesized that 5-ethynyl-2(1*H*)-pyrimidinone would have selectively inactivated liver DPD and spared intestinal DPD. The result from dosing rats with 5-ethynyl-2(1*H*)-pyrimidinone and 5-ethynyluracil demonstrated that 5-ethynyl-2(1*H*)-pyrimidinone was nearly as efficient an *in vivo* inactivator of DPD as 5-ethynyluracil (Table 1). Therefore, there was little tissue specificity for inactivation of DPD by 5-ethynyl-2(1*H*)-pyrimidinone. Thus, it appeared that either sufficient amounts of 5-ethynyl-2(1*H*)-pyrimidinone were oxidized and released systemically or there was sufficient aldehyde oxidase in the tissues studied to generate the necessary 5-ethynyluracil to inactivate DPD.

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