The Chemistry, Toxicology, and Identification in Rat and Human Urine of 4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one: A Reactive Metabolite in Felbamate Bioactivation

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Received June 30, 2000

4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one has been proposed to be a reactive metabolite of the anti-epileptic drug felbamate [Thompson et al. (1996) *Chem. Res. Toxicol.* **9**, 1225−1229]. 4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one exists in equilibrium with 3-oxo-2-phenylpropyl aminooate, which is known to eliminate to generate 2-phenylpropenal. Thus, this species is postulated to be a latent form of the ultimate reactive metabolite, 2-phenylpropenal. The chemistry of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one is proposed to parallel that of 4-hydroxycyclophosphamide, the bioactivated form of cyclophosphamide that undergoes ring-opening to aldophosphamide and subsequent elimination to afford 2-propenal (acrolein). The work presented here reports the chemical synthesis of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one and demonstrates that under buffered conditions it exists in equilibrium with 3-oxo-2-phenylpropyl aminooate. The rate-limiting step in the decomposition of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one is the irreversible β-elimination from 3-oxo-2-phenylpropyl aminooate to 2-phenylpropenal. We have found the half-life of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one to be 4.6 ± 0.4 h under in vitro conditions that mimic the physiological setting. As a consequence of the relatively long half-life of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one, we have sought evidence for the significance of this pathway in experimental and clinical conditions. We report here the observation of this metabolite in the urine of rats being treated with 3-hydroxy-2-phenylpropyl aminooate, the esterase-mediated metabolite of felbamate, and in the urine of patients undergoing felbamate therapy. In addition, we have shown that 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one is toxic to cultured cells in a time-dependent manner, most likely as a result of its decomposition to 2-phenylpropenal. Taken together, the data support the hypothesis that 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one represents a "time-release" form of 2-phenylpropenal capable of traveling to distal sites from its locus of bioactivation and thereby mediates felbamate associated toxicities.

Introduction

Felbamate (**1**), 3-(aminocarbonyloxy)-2-phenylpropyl aminooate, is an anti-epileptic drug useful in the treatment of several forms of epilepsy (*1, 2*). In its first year of widespread distribution, felbamate therapy was found to be associated with an increase in the incidence of aplastic anemia (*3*) and hepatotoxicity (*4*), resulting in its limited use. Previous research from our laboratory has shown that the primary metabolic pathway of felbamate proceeds through 3-oxo-2-phenylpropyl aminooate (**3**) (*5*). Under physiological conditions, 3-oxo-2-phenylpropyl aminooate has been shown to undergo both spontaneous cyclization to form 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one (**6**) and elimination to form 2-phenylpropenal (**5**), the proposed ultimate reactive metabolite in felbamate bioactivation (Scheme 1) (*5*). Additional research has identified *N*-acetylcysteine adducts of 2-phenylpropenal which are excreted in the urine of rats and humans after felbamate administration (*6, 7*). Given the reactivity of 2-phenylpropenal ($t_{1/2} \sim 40$ s in the presence of 500 μM glutathione), it is expected that 2-phenylpropenal will react at the site of formation. Therefore, the bioactivation of felbamate to 2-phenylpropenal in the liver could explain the observed hepatotoxicity, but is unlikely to account for the observed blood dyscrasias. We propose that 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one represents a latent form of 2-phenylpropenal capable of traveling to distal sites from the liver in the body.

The oxazolidine, 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one, exists in equilibrium with 3-oxo-2-phenylpropyl aminooate, which undergoes elimination to form 2-phenylpropenal (*5*). We propose that the essential features of the oxazolidine parallel the chemistry of 4-hydroxycyclophosphamide, which can undergo ring opening and elimination to 2-propenal (acrolein) (*8*). Studies have demonstrated that human serum albumin (HSA)[1] catalyzes the decomposition of 4-hydroxycyclo-

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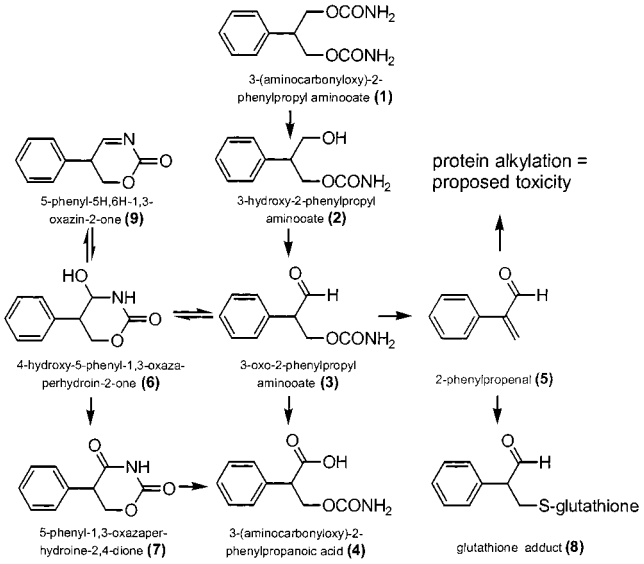
10.1021/tx000139n CCC: $20.00
Published on Web 07/07/2001

Scheme 1. The Metabolism of Felbamate



phosphamide (*9*) and may offer an explanation for the selective tissue toxicity of the oxazolidine. We hypothesize that the oxazolidine may reach the blood as a stable reservoir and "time release" form of 2-phenylpropenal and capable of meditating the observed blood dyscrasias.

There has been no direct observation of the oxazolidine in the blood or urine of patients being treated with felbamate. In vitro studies were able to demonstrate the generation of the oxazolidine by incubating 4-hydroxy-2-phenylpropyl aminooate (**2**) with S9 human liver fractions (*10*). In vivo work provided evidence for this metabolite by indirect observation of the corresponding oxazine dione (**7**) (*11*). The purpose of this work was to better characterize the chemistry and toxicology of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one and provide direct evidence for its formation in vivo.

Experimental Procedures

Chemicals and Instruments. The chemicals were purchased from either Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) and were of the highest grade available. The urinalysis was performed on a Waters 2690 Separations Module HPLC using a Waters Symmetry C_{18} (2.1 × 150 mm) column. The flow was directed into a Waters 486 Tunable absorbance detector set at $\lambda = 214$ nm and then into a Finnigan Mat LCQ ion trap atmospheric chemical ionization (APCI)[2] mass spectrometer. NMR spectra were obtained on a 300 MHz General Electric QE300 spectrometer, and chemical shifts are reported in ppm. The cell proliferation assays were read on a Molecular Devices Corporation Emax precision microplate reader set at $\lambda = 490$ nm.

Synthesis of 4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one (6). To a solution of 3-hydroxy-2-phenylpropyl aminooate (**2**) (100 mg, 0.512 mmol) in CH_2Cl_2 was added Dess−Martin periodinane (300 mg, 0.707 mmol). The mixture was stirred for 2 h and concentrated at room temperature. This reaction yielded 3-oxo-2-phenylpropyl aminooate (**3**), which was purified by flash chromatography using silica gel with ether. The aldehyde (**3**) was dissolved in acetone and an equal volume of 100 mM potassium phosphate buffer (pH 8.0). The mixture was stirred for 15 min and extracted with ethyl acetate, washed with brine, and dried with Na_2SO_4. The crude mixture was purified by flash chromatography using silica gel with ether, then ethyl acetate. Upon concentration, the ethyl acetate fractions afforded the product as pristine white crystals in good yield; >98% purity by HPLC. The product was isolated as a pair of diastereomers in a ratio of 3 (major epimer):1 (minor epimer) as determined by ^{1}H NMR. APCI-MS: parent $[M + 1H]^+$ $m/z = 194$. APCI-MS/MS: daughters $[M + 1H]^+$ $m/z = 150$ (loss of carbon dioxide) and $[M + 1H]^+$ $m/z = 133$ (2-phenylpropenal). Elemental analysis: theoretical C, 62.17; H, 5.74; N, 7.25; found C, 62.24; H, 5.81; N, 7.16. ^{1}H NMR (acetone-d_6): δ (major epimer) 2.72 (1H, bs), 2.96−3.04 (1H, m), 4.24−4.33 (2H, m), 5.04−5.11 (1H, d, $J = 5.39$ Hz), 6.73 (1H, bs), 7.15−7.32 (5H, m); (minor epimer) 2.68 (1H, bs), 3.26−3.34 (1H, m), 4.38−4.46 (2H, m), 5.20−5.25 (1H, d, $J = 6.16$ Hz), 6.73 (1H, bs), 7.15−7.32 (5H, m). ^{13}C NMR (acetone-d_6): δ 43.90, 67.65, 77.75, 128.00, 128.14, 129.3, 137.33, 155.27.

Synthesis of 3-Oxo-2-phenylpropyl Aminooate (3). The aldehyde metabolite was synthesized from 3-hydroxy-2-phenylpropyl aminooate as follows. To a solution of 3-hydroxy-2-phenylpropyl aminooate (100 mg, 0.51 mmol) in 10 mL of CH_2Cl_2 was added Dess−Martin periodinane (300 mg, 0.71 mmol) in an aluminum foil covered round-bottom flask. After stirring for 2 h at room temperature, the mixture was separated immediately using silica gel (ether, $R_f = 0.7$). The solvent was removed in vacuo to provide the product as solid white crystals (94 mg, 95%). The product is stable at −80 °C, neat or as a solution in dry acetone. At room temperature, the product readily decomposes to 2-phenylpropenal and its dimerization product, which is yellow in color. The ^{1}H NMR (acetone-d_6) agreed with the previously published data (*5*). The compound purity was determined to be ≥95% pure as determined by HPLC/UV at $\lambda = 214$ nm. A slight impurity of 2-phenylpropenal was observed in the sample and background corrections are included in the reported data.

The pH-Dependent Decomposition of 4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one (6). The oxazolidine (**6**, 100 μM) was incubated at 37 °C in 100 mM potassium phosphate buffer at various pH units. For incubations with HSA (fatty acid and globulin free; Sigma A-3782), HSA was added to a final concentration of 60 mg/mL. At each time point, 50 μL of the sample was removed and mixed with 50 μL of 100 μM felbamate (**1**) as an internal standard. The amount of oxazolidine remaining was determined by HPLC [Waters Symmetry C_{18} column 2.1 × 150 mm; 200 μL/min flow rate; 30% CH_3CN:70%-(0.1%)HOAc; UV detection, $\lambda = 214$ nm]. The half-life was determined using a first-order approximation and plotting ln [**6**] versus time. For each pH value, the experiments were run in triplicate.

The Rate-Limiting Step in 4-Hydroxy-5-phenyltetrahydro-1,3-oxazin-2-one Decomposition. To determine the rate-limiting step in 4-hydroxy-5-phenyltetrahydro-1,3-oxazin-2-one decomposition, we trapped the aldehyde intermediate using *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride at pH 5. The trapping experiment was conducted at pH 5 because of the pH optimum for the reaction. To 950 μL of phosphate buffer, pH 5, at 37 °C was added 50 μL of 20 mM *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine in ethanol and then 5 μL of 20 mM 4-hydroxy-5-phenyltetrahydro-1,3-oxazin-2-one. At each time point, 50 μL of the sample was removed and mixed with 50 μL of 100 μM felbamate as an internal standard. The amount of **6** remaining was determined by HPLC [Waters Symmetry C_{18} column 2.1 × 150 mm; 200 μL/min flow rate; 30% CH_3CN: 70%(0.1%)HOAc; UV detection, $\lambda = 214$ nm]. The half-life was determined using a first-order approximation and plotting ln [**6**] versus time. For each pH value, the experiments were run in triplicate.

The Decomposition of 3-Oxo-2-phenylpropylaminooate (3). The aldehyde carbamate was dissolved in acetone on dry ice to a final concentration of 50 mM. The aldehyde carbamate solution was then diluted into phosphate buffer at various pH units at 37 °C to 350 μM. Immediately after diluting the aldehyde into the buffer, an aliquot was removed, felbamate was added as an internal standard (100 μM), and 15 μL was injected

[1] HSA: human serum albumin.
[2] APCI: atmospheric pressure chemical ionization.

onto the HPLC column. The amount of the oxazolidine and 2-phenylpropenal formed at ~1 min was determined by HPLC [Waters Symmetry C_{18} column 2.1 × 150 mm; 200 μL/min flow rate; 30% CH_3CN:70%(0.1%)HOAc; UV detection, $\lambda = 214$ nm]. Calibration curves for the oxazolidine and 2-phenylpropenal using felbamate as an internal standard were determined and found to be linear. Under acidic conditions (pH 5) and about 1 min, a significant amount of 3-oxo-2-phenylaminooate remained, and this suggests that the aldehyde and the oxazolidine had not reached equilibrium. In contrast, under physiological conditions, we did not observe the aldehyde after about 1 min of incubation, which suggests that the aldehyde and oxazolidine had reached equilibrium.

Identification of 4-Hydroxy-5-phenyl-1,3-oxaza-perhydroin-2-one (6) in Rat and Human Urine. The animal experiments were conducted under a protocol approved by the University of Virginia Animal Use and Care Committee (#2799-05-98), and the patient urine was collected under a protocol approved by the University of Virginia Human Investigations Committee (#7310). Rat urine was collected continuously for 18 h post dose from 250 g Sprague−Dawley rats that had been dosed chronically for 2 weeks with 70 mg kg^{-1} day^{-1} of 3-hydroxy-2-phenylpropyl aminooate or control rats that had not been treated. The rat urine was stored at −60 °C until thawed at 37 °C at the time of analysis. The human urine was obtained from patients undergoing felbamate therapy and analyzed about 18 h post collection. The control urine samples were collected from adult volunteers not being treated with felbamate. The human samples were stored at room temperature. The urine (3 mL for rat; 50 mL for human) was extracted 3 times with an equal volume of ethyl acetate and dried with Na_2SO_4. The solvent was removed by rotary evaporation, and the solid was dissolved immediately in 500 μL of acetone on dry ice. The acetone solution (50 μL) was diluted into 150 μL of 0.1% HOAc in water and analyzed by LC/MS/MS. Three MCF-dosed rat urine samples, one blank rat urine sample, four patient urine samples, and three blank urine samples were analyzed in total. The reported results correspond to representative analysis. For the coelution experiments, the oxazolidine synthetic standard was added to the extracted sample to give a final concentration of 10 μM, except in the reported MCF-dosed rat coelution, in which case synthetic standard was added to a final concentration of 50 μM because of the high level of the oxazolidine present. The sample (15 μL) was analyzed by HPLC (as above) and detected with positive ion APCI-MS/MS (Finnigan LCQ). The ion trap was set to trap the parent ion ($m/z = 194$) with a 3.0 m/z isolation width. Fragmentation was achieved with 16% collision energy using helium gas, and daughter ions were detected in the full scan mode from 100 to 200 m/z. The tune parameters were as follows: source voltage = 4.14 kV; source current = 4.83 μA; vaporizer temperature = 450 °C; sheath gas (nitrogen) = 34.9; auxiliary gas (nitrogen) = 29.2; capillary voltage = 2.85 V; capillary temperature = 150 °C.

Growth Inhibition Assays (GI_{50}). The cells used for the growth inhibition studies were HEK-293/SF (human kidney) obtained from the American Type Culture Collection (Manassas, VA) that had been adapted to grow in serum-free media. The cell line was selected because it was established as a serum-free cell line. Serum culture conditions were avoided because of the potential for serum proteins to react with the compounds of interest. The cells were grown in Nephrigen Serum Free Media obtained from Celox Laboratories, Inc. (St. Paul, MN), at 37 °C in humidified air containing 5% carbon dioxide. For the assays containing fetal bovine serum (FBS), FBS was added to the Nephrigen Serum Free Media to a final concentration of 10% (v/v). The cells were plated into a 96-well plate at a density of 1×10^4 cells per well and allowed to adhere to the plates for 24 h. After 24 h, the medium was removed and replaced with media containing 25, 12.5, 10, 7.5, 5.0, or 2.5 μM of each drug (**6**, **5**, and 2-propenal) with less than 0.5% EtOH (v/v). Control experiments showed that 0.5% EtOH has no effect on cell survival. The cells were allowed to grow in the drug-containing medium for 72 h, at which time the medium was removed and the number of active cells was determined using the Promega (Madison, WI) MTS cell proliferation assay. Briefly, the cells were incubated with 100 μL of RPMI media (Life Technologies, Gaithersburg, MD) containing 2.5 nmol of phenazime methosulfate (PMS) and 66 nmol of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) for 1 h. MTS is bioreduced in the presence of PMS by dehydrogenase enzymes in viable cells to a formazan, which absorbs light at 490 nm. The absorbance at 490 nm is directly proportional to the number of active cells. The GI_{50} for each drug was determined by plotting the percent growth inhibition versus drug concentration in the linear region, determining the linear regression for each plot, and calculating the GI_{50} based on the linear regression. For each compound, the assay was performed in quadruplicate, and the data are reported as the average of those assays ± the standard deviation.

Time-Dependent Growth Inhibition. The HEK293/SF cells (as above) were plated into 96-well plates at a density of 1×10^4 cells per well and allowed to adhere for 24 h. After 24 h, the medium was removed and replaced with 50 μM drug (**6** or **5**)-containing serum-free media. At each time point, the drug-containing medium was removed, and the number of viable cells was determined using the Promega MTS cell proliferation assay described above. The assays were performed in quadruplicate.

Results

Synthesis of 4-Hydroxy-5-phenyltetrahydro-1,3-oxazain-2-one. The oxazolidine was synthesized and characterized as reported under Experimental Procedures. The synthesis afforded the desired product and provides a route to producing several grams of material. We found the oxazolidine to be stable neat at room temperature for several months and dissolved in acetonitrile at −20 °C for several months. In aqueous solutions, the oxazolidine readily equilibrates to 3-oxo-2-phenylpropyl aminooate and eventually eliminates to afford 2-phenylpropenal.

The pH-Dependent Decomposition of 4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one. The decomposition of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one to 2-phenylpropenal occurs spontaneously in aqueous solutions. The decomposition demonstrates base catalysis and has a half-life of 4.6 ± 0.4 h under physiological conditions (Figure 1). Given time, the oxazolidine decomposes to produce one product, 2-phenylpropenal. The addition of physiologically relevant concentrations of HSA accelerated the rate of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one decomposition to 50 ± 3 min when monitoring the loss of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one. Formation of 2-phenylpropenal was not observed and is thought to be binding to HSA (Table 1).

The Rate-Limiting Step in the Decomposition of 4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one. Trapping 3-oxo-2-phenylpropyl aminooate during the decomposition of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one would allow for the determination of the rate-limiting step in the decomposition of the oxazolidine. If the ring opening from the oxazolidine to 3-oxo-2-phenylpropyl aminooate is the rate-determining step in its decomposition, trapping the aldehyde carbamate should not change the oxazolidine decomposition half-life. However, if the β-elimination from 3-oxo-2-phenylpropyl aminooate to 2-phenylpropenal is the rate-limiting step in the oxazolidine decomposition, trapping the aldehyde carbamate should result in a decrease in the half-life of

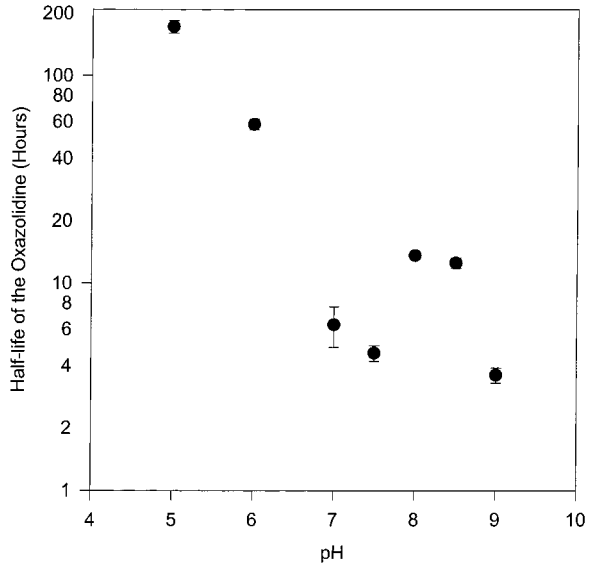


Figure 1. The pH-dependent decomposition of 4-hydroxy-5-tetrahydro-1,3-oxazin-2-one (**6**). At pH 12, the oxazolidine half-life was less than 15 min. Error bars are included for each data point.

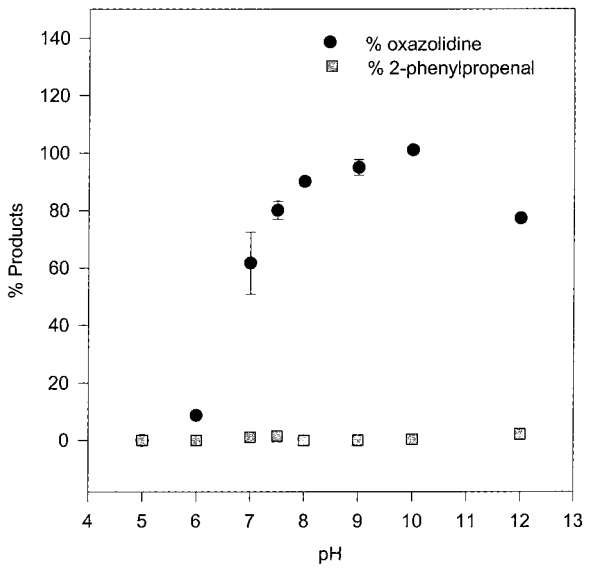


Figure 2. The pH-dependent product formation from 3-oxo-2-phenylpropyl aminooate at ~1 min.

Table 1. The Half-Life of 4-Hydroxy-5-tetrahydro-1,3-oxazin-2-one under Physiologically Relevant Conditions

conditions	$T_{1/2}$ (h)
100 mM K_2HPO_4, pH 7.5	4.6 ± 0.4
100 mM K_2HPO_4, pH 7.5, + 60 mg of HSA/mL	0.8 ± 0.1

the oxazolidine. The trapping experiment caused a decrease in the half-life of the oxazolidine at pH 5 from 172 ± 12 to 57 ± 9 h. The data support the hypothesis that the oxazolidine and aldehyde carbamate exist in an equilibrium that is rapid relative to the rate-limiting step in the oxazolidine decomposition. The rate-limiting step is the β-elimination reaction from the aldehyde carbamate that affords 2-phenylpropenal. The kinetics governing the oxazolidine decomposition mimic the kinetics observed for the decomposition of 4-hydroxycylophosphamide in that 4-hydroxycyclophosphamide has also been shown to exist in a rapid equilibrium with aldophosphamide with rate-limiting β-elimination affording 2-propenal (*12*).

The Decomposition of 3-Oxo-2-phenylpropyl Aminooate. The interesting increase in the half-life of the oxazolidine metabolite in the slightly basic region prompted us to address the decomposition rate of the aldehyde carbamate, 3-oxo-2-phenylpropyl aminooate, to afford the oxazolidine and 2-phenylpropenal. Previous data had reported that the decomposition of the aldehyde carbamate occurs in a constant 10:1 ratio (oxazolidine to 2-phenylpropenal, respectively) throughout an acidic, neutral, and basic pH region (*5*). We propose that the increase in the half-life of the oxazolidine metabolite in the slightly basic region occurs as a result of general acid/base catalysis, which is not consistent with the previously reported constant 10:1 ratio. Our results conflict with the prior data and demonstrate that the decomposition of the aldehyde carbamate does not decompose to the oxazolidine metabolite and 2-phenylpropenal in a constant ratio throughout the pH-buffered region examined (Figure 2). The data reported here demonstrate that the oxazolidine metabolite has a pH optimum for formation from the aldehyde carbamate in the slightly basic pH region. The newly reported data are consistent with general acid/base catalysis for oxazolidine formation from 3-oxo-2-phenylpropyl aminooate and offer an explanation for the increased half-life of the oxazolidine in the slightly alkali region.

Identification of 4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one in Patient and 3-Hydroxy-2-phenylpropyl Aminooate Dosed Rat Urine. Given the relatively long half-life, we sought to identify 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one in the urine of patients being treated with felbamate and rats that had been dosed with the esterase-mediated felbamate metabolite, 3-hydroxy-2-phenylpropyl aminooate. We sought to identify this metabolite in rats that had been treated with 3-hydroxy-2-phenylpropyl aminooate, as opposed to felbamate, given the significant interspecies metabolism differences (*13*). The oxazolidine metabolite was identified routinely in both the patient urine and rat urine by LC/MS/MS coelution experiments. Under the conditions applied, the oxazolidine metabolite elutes at 3.3 min and produced characteristic MS/MS spectra with daughter ions $m/z = 176$ (loss of water), $m/z = 150$ (loss of carbon dioxide), $m/z = 133$ (2-phenylpropenal), and $m/z = 132$ (2-phenylpropenal preformed cation) (Figures 3a−c and 4a−c).

The Growth Inhibition of 4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one on Cells in Culture. The oxazolidine metabolite, 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one, produced 50% growth inhibition at 13.0 ± 1.4 μM when applied to HEK-293 cells as compared to 5.4 ± 1.8 μM for 2-phenylpropenal and 10.7 ± 1.7 μM for 2-propenal. Control experiments showed that 0.5% ethanol had no effect on growth inhibition.

The Time-Dependent Growth Inhibition of 4-Hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one and 2-Phenylpropenal. The growth inhibition of 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one most likely results from its decomposition to 2-phenylpropenal, and not as a result of inherent properties. Within the first hour of incubation on HEK-293 cells under serum-free conditions, 2-phenylpropenal caused almost 100% growth inhibition (Figure 5). The growth inhibition of the ox-

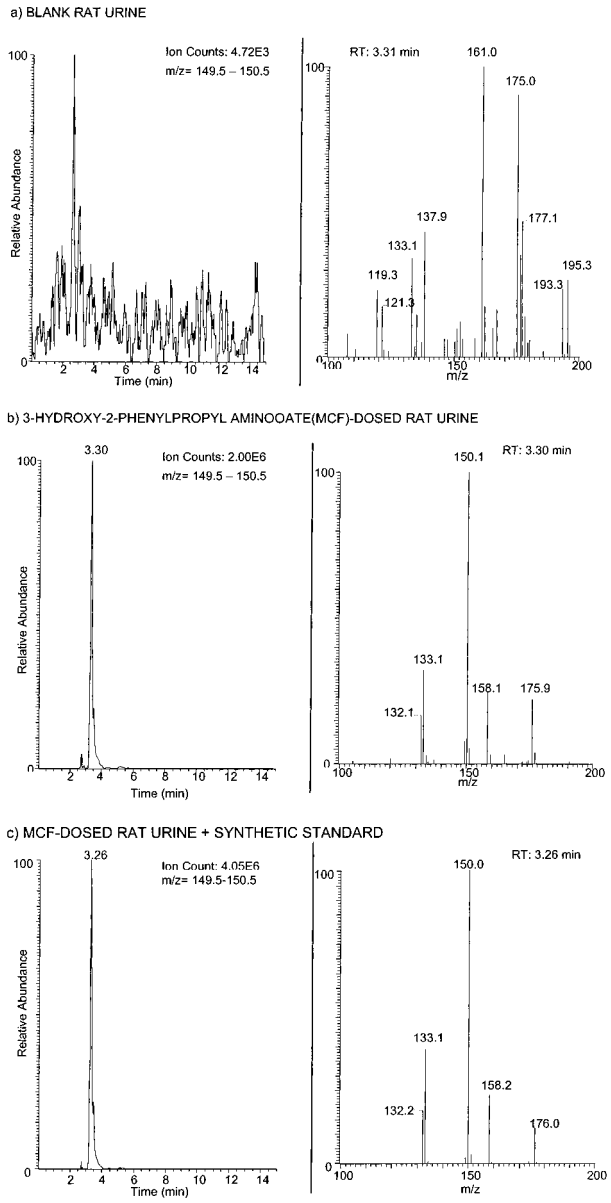


Figure 3. (a) Blank rat urine. Above left shows the LC chromatograph with mass filter $m/z = 150$ for a characteristic daughter ion of 4-hydroxy-5-tetrahydro-1,3-oxazin-2-one (**6**) ($m/z = 194$). Above right shows the MS/MS spectrum (RT = 3.31 min from LC chromatograph on left) with no detectable daughter ions characteristic for the oxazolidine metabolite. (b) Identification of 4-hydroxy-5-tetrahydro-1,3-oxazin-2-one (**6**) in rat urine extract. Data are displayed as in (a). Above right shows the MS/MS spectrum (RT = 3.30 min from LC chromatograph on left) of daughters $m/z = 176$ (loss of water), $m/z = 158$ (loss of CO_2), $m/z = 150$ (loss of CO_2 and NH_3), $m/z = 133$ (2-phenylpropenal), and $m/z = 132$ (2-phenylpropenal preformed cation). (c) Rat urine extract with synthetic standard. Data are displayed as in (a). The coelution of the synthetic standard with the rat urine extract was indicated by an increase in total ion count and the same retention time, as shown in (b).

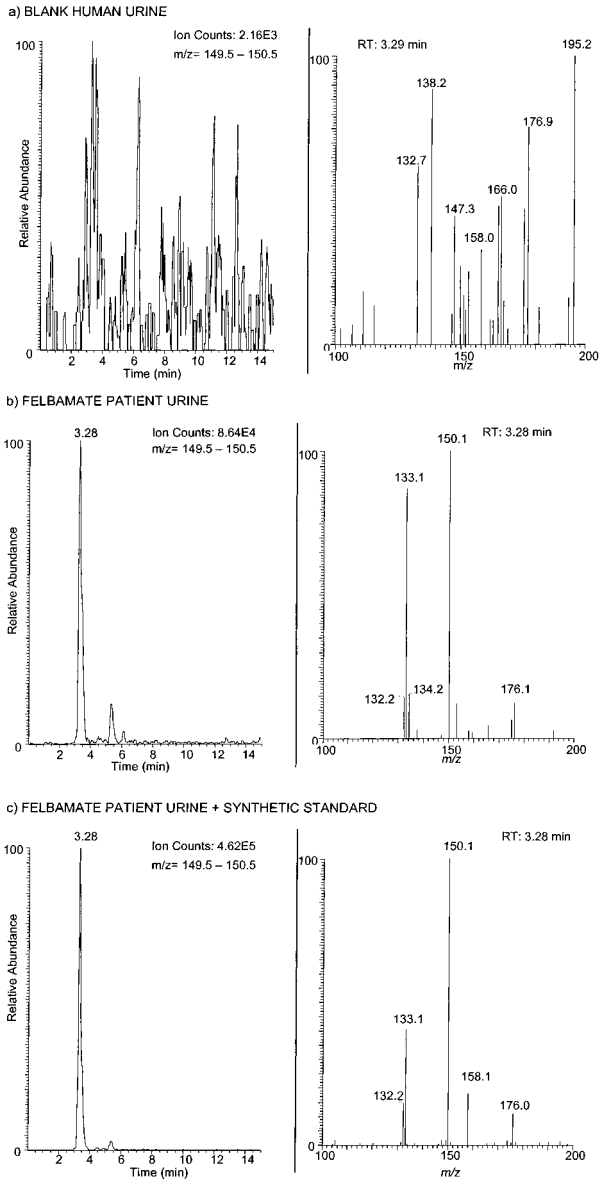


Figure 4. (a) Blank human urine. Above left shows the LC chromatograph with mass filter $m/z = 150$ for a characteristic daughter ion of 4-hydroxy-5-tetrahydro-1,3-oxazin-2-one (**6**) ($m/z = 194$). Above right shows the MS/MS spectrum (RT = 3.29 min from LC chromatograph on left) with no detectable daughter ions characteristic for the oxazolidine metabolite. (b) Identification of 4-hydroxy-5-tetrahydro-1,3-oxazin-2-one (**6**) in patient urine extract. Data are displayed as in (a). Above right shows the MS/MS spectrum (RT = 3.28 on left) of daughters $m/z = 176$ (loss of water), $m/z = 158$ (loss of CO_2), $m/z = 150$ (loss of CO_2 and NH_3), $m/z = 133$ (2-phenylpropenal), and $m/z = 132$ (2-phenylpropenal preformed cation). (c) Human urine extract with synthetic standard. Data are displayed as in (a). The coelution of the synthetic standard with the patient urine extract was indicated by an increase in the total ion count and the same retention time, as indicated in (b).

azolidine occurs over time and interestingly produces about 50% growth inhibition at about 5 h (Figure 5) or the half-life of **6** under physiological conditions (Figure 1).

Discussion

The hypothesis that 4-hydroxy-5-phenyl-1,3-oxazaperhydroin-2-one, an oxazolidine metabolite of felbamate, represents a latent form of 2-phenylpropenal capable of travelling to sites distal from the liver requires that it "has a life" in vivo. To this end, we studied the half-life of the oxazolidine throughout a phosphate buffer pH range. We expected that both the ring opening of the oxazolidine to 3-oxo-2-phenylpropyl aminooate and β-elimination of the 3-oxo-2-phenylpropyl aminooate to 2-phenylpropenal would undergo general base catalysis. Our results (Figure 1) have confirmed that the oxazolidine, which exists in a rapid equilibrium with 3-oxo-2-phenylpropyl aminooate, undergoes base-catalyzed de-

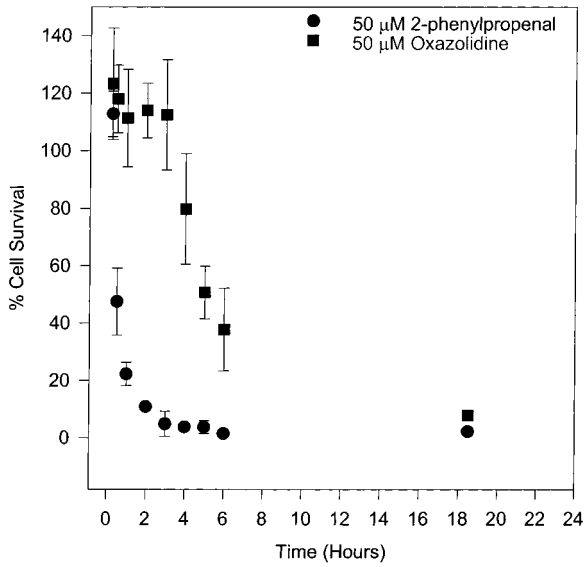


Figure 5. The time-dependent cytotoxic effect of 4-hydroxy-5-tetrahydro-1,3-oxazin-2-one (**6**) probably as a result of its decomposition to 2-phenylpropenal (**5**). The compound 2-phenylpropenal (**5**) demonstrates its maximal toxicity within 1 h of drug exposure.

composition to 2-phenylpropenal. To determine the rate-limiting step in the decomposition of the oxazolidine to 2-phenylpropenal, we studied the effect of trapping 3-oxo-2-phenylpropyl aminooate on the half-life of the oxazolidine decomposition. We trapped 3-oxo-2-phenylpropyl aminooate using *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride under acidic conditions, pH 5. Under these conditions, trapping 3-oxo-2-phenylpropyl aminooate decreased the half-life of the oxazolidine from 172 ± 12 to 57 ± 9 h. The observed decrease in the half-life of the oxazolidine by trapping 3-oxo-2-phenylpropyl aminooate is consistent with the proposal that the oxazolidine and 3-oxo-2-phenylpropyl aminooate exist in an equilibrium that is rapid relative to the β-elimination, and that the β-elimination affording 2-phenylpropenal is rate-limiting.

The increase in the oxazolidine half-life in the slightly basic region was thought to result from the cyclization kinetics of 3-oxo-2-phenylpropyl aminooate as governed by general acid/base catalysis (Figure 1). However, given this proposal, the cyclization should demonstrate a pH optimum in the slightly basic region. This proposal was inconsistent with previous data, which demonstrated that upon incubation throughout a pH-buffered range, 3-oxo-2-phenylpropyl aminooate cyclizes to form the oxazolidine and eliminates to form 2-phenylpropenal in a constant 10:1 ratio, respectively (*5*). Since the previous data were inconsistent with our hypothesis regarding the kinetics of the oxazolidine decomposition, we repeated the study to monitor the formation of the oxazolidine and 2-phenylpropenal from 3-oxo-2-phenylpropyl aminooate at various pH units. Our results conflict with the previously published data and demonstrate that the ratio of the oxazolidine to 2-phenylpropenal formation is not a constant ratio (Figure 2). The data demonstrate general acid/base catalysis for the oxazolidine formation from 3-oxo-2-phenylpropyl aminooate and general base catalysis for the β-elimination of 3-oxo-2-phenylpropyl aminooate to 2-phenylpropenal. The increase in the half-life of the oxazolidine in the slightly basic region reflects a pH optimum for the oxazolidine formation from 3-oxo-2-phenylpropyl aminooate. We estimate that for 3-oxo-2-phenylpropyl aminooate, the pK_a of the carbamate proton is ~12 and the pK_a of the α-carbon proton is ~16 (based on reported literature values in water) (*14*). The differences in pK_a values explain the preference for cyclization over elimination as demonstrated. Under physiological conditions, 3-oxo-2-phenyl aminooate rapidly cyclizes, generating the oxazolidine metabolite almost exclusively. Given the equilibrium between the oxazolidine and 3-oxo-2-phenylpropyl aminooate and the irreversible formation of 2-phenylpropenal from 3-oxo-2-phenylpropyl aminooate, the oxazolidine completely decomposes to 2-phenylpropenal over time. Under physiological conditions, the oxazolidine demonstrates a half-life of 4.6 h, which is sufficient time to travel to sites distal from the liver. The oxazolidine could "have a life" in vivo.

The comparison of the oxazolidine metabolite to 4-hydroxycyclophosphamide led us to consider other possible equilibrium species, including the dehydration of the oxazolidine to afford an imine and the hydrolysis of 3-oxo-2-phenylpropyl aminooate to afford the hydrate (*12*, *15*−*17*). The dehydration of carbinolamines depends on both the protonation of the leaving group and the deprotonation of the amine, resulting in optimum imine formation under slightly alkali conditions (*18*). Maximal imine formation is expected to occur under slightly basic conditions and may contribute to the increase in the half-life of the oxazolidine in this pH region. Hydrate formation of the aldehyde carbamate would be expected to occur under acidic conditions; we hypothesize that hydrate formation may contribute to the stability of the aldehyde carbamate under acidic conditions. We suspect that each of these equilibrium species may contribute to the overall in vivo stability of the oxazolidine.

Incubating the oxazolidine with HSA decreased the oxazolidine half-life to 50 min (Table 1). The accelerated decomposition of the oxazolidine in the presence of HSA may provide insight into the hypothesis that the oxazolidine represents a latent form of 2-phenylpropenal that may be released selectively in the blood to mediate the observed blood dyscrasias. In the same incubations, we were unable to observe the formation of 2-phenylpropenal from the oxazolidine metabolite and hypothesize that 2-phenylpropenal is binding to HSA. HSA contains a free thiol at Cys-34 that has been shown to react with several compounds including the bio-activated forms D-penicillamine, captopril, benzene, and acetaminophen (*19*). The reactive Cys-34 resides in a hydrophobic crevice of depth 9.5−10 Å (*19*). We estimate that 2-phenylpropenal is small enough to fit into this binding pocket and propose that 2-phenylpropenal may undergo a Michael addition to HSA. The alkylation of HSA by 2-phenylpropenal represents a potential hapten that could illicit immune-mediated toxicity, which is consistent with the hapten hypothesis of idiosyncratic drug reactions. In cases of other drug toxicities including nitrofurantoin and penicillin, albumin-related antibodies have been observed (*19*).

Given the significant half-life of the oxazolidine, we sought to identify this metabolite in the urine of rats being treated with 3-hydroxy-2-phenylpropyl aminooate and patients being treated with felbamate. The pH of urine was found to be about 6 for rats and slightly more acidic, or about 5, for humans. The oxazolidine was not detected in control rat urine (Figure 3a) or control human urine (Figure 4a) but was observed routinely in the urine of rats treated with the esterase-mediated felbamate

metabolite, 3-hydroxy-2-phenylpropyl aminooate (Figure 3b,c), and in felbamate patient urine samples (Figure 4b,c). While another laboratory has demonstrated the formation of the oxazolidine in vitro (*10*), these data represent the first direct evidence for its formation in vivo.

To further characterize the oxazolidine, we examined its toxicity in vitro. We determined a GI_{50} for the oxazolidine on HEK-293 cells cultured under serum-free conditions. Serum-free conditions were employed due to the high reactivity of 2-phenylpropenal and the possibility of protein binding. While not quite as toxic as 2-phenylpropenal ($GI_{50} = 5.4 \pm 1.8\ \mu M$) or 2-propenal ($GI_{50} = 10.7 \pm 1.7 \mu M$), the oxazolidine ($GI_{50} = 13.0 \pm 1.4\ \mu M$) demonstrates toxicity within the same order of magnitude. It is worth noting that 2-phenylpropenal appears to be slightly more toxic than acrolein probably as a consequence of increased electrophilicity introduced by the addition of the phenyl ring. The increased toxicity of 2-phenylpropenal as compared to acrolein is consistent with previously published results on HA1 cells (*5*). Since kidney cells are metabolically active, the slight increase in GI_{50} for the oxazolidine is likely to result in its oxidation to tetrahydro-1,3-oxazin-2,4-dione (**7**) or oxidation of 3-oxo-2-phenylpropyl aminooate to 3-carbamoyl-2-phenylpropionic acid. To study the possible effect of protein binding, the same experiments were conducted with media supplemented with fetal bovine serum (FBS) to 10% (v/v). Interestingly, the addition of FBS had no effect on the GI_{50} of either the oxazolidine or the 2-phenylpropenal.

To support the hypothesis that the toxicity of the oxazolidine results from its decomposition to 2-phenylpropenal, we conducted time-dependent growth inhibition experiments. The time-dependent growth inhibition study was able to demonstrate that 2-phenylpropenal causes almost 100% growth inhibition within the first hour of drug exposure (Figure 5). The results indicate that 2-phenylpropenal-induced growth inhibition is not cell cycle dependent. The growth inhibition of the oxazolidine was found to be time-dependent and demonstrates 50% growth inhibition between 4 and 5 h of incubation. Furthermore, the time-dependent growth inhibition data correlate nicely with the observed half-life of the oxazolidine under physiological conditions (~4.6 h) (Figure 1). While the data are not conclusive, they are consistent with the hypothesis that the oxazolidine-mediated growth inhibition results as a consequence of its decomposition to 2-phenylpropenal.

The chemistry, toxicology, and identification of the oxazolidine in vivo support our hypothesis that it represents a latent precursor of 2-phenylpropenal capable of travelling to sites distal from the liver in the body. We believe these characteristics of the oxazolidine may contribute importantly to the observed felbamate-induced blood dyscrasias. The commonalities between the chemistry of the oxazolidine and 4-hydroxycyclophosphamide offer insights into the reactivity of compounds with similar structural motifs and possibly to their selective blood cell toxicity.

Acknowledgment. We thank Wallace Laboratories for generous financial support for this research.

References

(1) Fraser, A. D. (1996) New drugs for the treatment of epilepsy. *Clin. Biochem.* **29**, 97−110.

(2) Leppik, I. E. (1996) Felbamate. in *The Treatment of Epilepsy* (Shorvon, S., Dreifuss, F., Fish, D., and Thomas, D., Eds.) pp 421−428, Blackwell Science, New York.

(3) Pennell, P. B., Ogaily, M. S., and Macdonald, R. L. (1995) Aplastic anemia in a patient receiving felbamate for complex partial seizures. *Neurology* **45**, 456−460.

(4) O'Neil, M. G., Perdun, C. S., Wilson, M. D., McGown, S. T., and Patel, S. (1996) Felbamate-associated fatal acute hepatic necrosis. *Neurology* **46**, 1457−1459.

(5) Thompson, C. D., Kinter, M. T., and Macdonald, T. L. (1996) Synthesis and in vitro reactivity of 3-carbamoyl-2-phenylpropionaldehyde and 2-phenylpropenal: putative reactive metabolites of felbamate. *Chem. Res. Toxicol.* **9**, 1225−1229.

(6) Thompson, C. D., Gulden, P. H., and Macdonald, T. L. (1997) Identification of modified atropaldehyde mercapturic acids in rat and human urine after felbamate administration. *Chem. Res. Toxicol.* **10**, 457−462.

(7) Thompson, C. D., Barthen, M. T., Hopper, D. W., Miller, T. A., Quigg, M., Hudspeth, C., Montouris, G., Marsh, L., Perhach, J. L., Sofia, R. D., and Macdonald, T. L. (1999) Quantification in patient urine samples of felbamate and three metabolites: acid carbamate and two mercapturic acids. *Epilepsia* **40**, 769−776.

(8) Sladek, N. E. (1988) Metabolism of oxazphosphorines. *Pharmacol. Ther.* **37**, 301−355.

(9) Kwon, C., Maddison, K., LoCastro, L., and Borch, R. F. (1987) Accelerated decomposition of 4-hydroxycyclophosphamide by human serum albumin. *Cancer Res.* **47**, 1505−1508.

(10) Kapetanovic, I. M., Torchin, C. D., Thompson, C. D., Miller, T. A., McNeilly, P. J., Macdonald, T. L., Kupferberg, H. J., Perhach, J. L., Sofia, R. D., and Strong, J. M. (1998) A potentially reactive cyclic carbamate metabolite of the anti-epileptic drug felbamate produced by human liver tissue in vitro. *Drug Metab. Dispos.* **26**, 1089−1085.

(11) Thompson, C. D., Miller, T. A., Barthen, M. T., Dieckhaus, C. M., Sofia, R. D., and Macdonald, T. L. (2000) The synthesis, in vitro reactivity, and evidence for formation in humans of 5-phenyl-1,3-oxazinane-2,4-dione, a metabolite of felbamate. *Drug Metab. Dispos.* **28**, 434−439.

(12) Borch, R. F., Hoye, T. R., and Swanson, T. A. (1984) In situ preparation of *cis*- and *trans*-4-hydroxycyclophosphamide with aldophosphamide and its hydrate in aqueous solution. *J. Med. Chem.* **27**, 490−494.

(13) Dieckhaus, C. M., Miller, T. A., Sofia, R. D., and Macdonald, T. L. (2000) A mechanistic approach to understanding species differences in felbamate bioactivation: relevance to drug-induced idiosyncratic reactions. *Drug Metab. Dispos.* **28**, 814−822.

(14) Lide, D. R., Ed. (1999) *CRC Handbook of Chemistry and Physics*, 80th ed., pp 8-46−8-56, CRC Press, New York.

(15) Fenselau, C., Lehman, J. P., Myles, A., Brandt, J., Yost, G. S., Friedman, O. M., and Colvin, O. M. (1982) Iminocyclophosphamide as a chemically reactive metabolite of cyclophosphamide. *Drug Metab. Dispos.* **10**, 636−640.

(16) Borch, R. F., and Getman, K. M. (1984) Base-catalyzed hydrolysis of 4-hydroxycyclophosphamide: evidence for iminocyclophosphamide as an intermediate. *J. Med. Chem.* **27**, 485−490.

(17) Borch, R. F., and Millard, J. A. (1987) The mechanism of activation of 4-hydroxycyclophosphamide. *J. Med. Chem.* **30**, 427−431.

(18) Sayer, J. M., Peskin, M., and Jencks, W. P. (1973) Imine-forming elimination reactions. I. General base and acid catalysis and influence of the nitrogen substituent on rates and equilibria for carbinolamine dehydration. *J. Am. Chem. Soc.* **95**, 4278−4287.

(19) Peters, T. (1996) *All About Albumin*, Academic Press, New York.

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