Cimetidine Inhibition of Human Gastric and Liver Alcohol Dehydrogenase Isoenzymes: Identification of Inhibitor Complexes by Kinetics and Molecular Modeling†

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ABSTRACT: Cimetidine, an H_2-receptor antagonist, is one of the most commonly prescribed drugs in the world. It has been reported to increase blood alcohol concentrations in drinking individuals. To determine if this increase could be due to inhibition of alcohol dehydrogenase activity, the effect of the drug on ethanol oxidation by gastric $\sigma\sigma$ alcohol dehydrogenase and liver $\beta_2\beta_2$, $\pi\pi$, and $\chi\chi$ alcohol dehydrogenase isoenzymes was observed. Cimetidine inhibited all isoenzymes studied except $\chi\chi$; the $\chi\chi$ isoenzyme showed no inhibition up to 5 mM cimetidine. Inhibition of the alcohol dehydrogenase isoenzymes by the H_2-receptor antagonists nizatidine, ranitidine, and famotidine was negligible. Docking simulations with the β_2•NAD^+•4-iodopyrazole X-ray structure indicated that cimetidine fit well into the substrate binding site. The substitution on the thiazole ring of nizatidine, however, prevented docking into the binding site. Cimetidine inhibition of ethanol oxidation by $\sigma\sigma$ and $\beta_2\beta_2$ was competitive with varied ethanol, exhibiting K_i values of 2.8 ± 0.4 mM and 0.77 ± 0.07 mM, respectively. Cimetidine inhibition of ethanol oxidation by $\pi\pi$ was noncompetitive with varied ethanol (K_i = 0.50 ± 0.03 mM). Inhibition of ethanol oxidation by $\sigma\sigma$ and $\beta_2\beta_2$ with varied NAD^+ was competitive. These results, together with the cimetidine inhibition kinetics of acetaldehyde reduction by $\sigma\sigma$ and $\beta_2\beta_2$, with either varied NADH or varied acetaldehyde, are consistent with cimetidine binding to two enzyme species. These species are free enzyme and the productive enzyme•NAD^+ complex.

Ethanol oxidation in human tissue is predominantly catalyzed by NAD^+-dependent alcohol dehydrogenase isoenzymes. The isoenzymes are active as dimers, and human liver expresses a variety of subunits, grouped into class I (α, β, and γ),[1] class II (π), and class III (χ) (Ehrig et al., 1990). Human stomach mucosa contains the $\gamma\gamma$ and $\chi\chi$ isoenzymes, as well as the class IV $\sigma\sigma$ (Moreno & Parés, 1991), also known as gastric or μ-alcohol dehydrogenase (Yin et al., 1993). The purified human liver and gastric alcohol dehydrogenase isoenzymes exhibit different V_{max} and K_m values for ethanol oxidation (Ehrig et al., 1990; Stone et al., 1993a). All isoenzymes appear to function by the ordered Bi-Bi mechanism (Burnell & Bosron, 1989; Kedishvili et al., 1994). This mechanism dictates that NAD^+ or NADH binds to the enzyme before the alcohol or aldehyde substrate can bind. The alcohol dehydrogenase isoenzymes contain two zinc atoms per subunit (Eklund & Brändén, 1979; Moreno & Parés, 1991); one is considered structural, while the other, located in the active site, is vital to enzyme activity.

Cimetidine, an H_2-receptor antagonist used to treat peptic ulcers, is reportedly an inhibitor of gastric alcohol dehydrogenase ethanol-oxidizing activity (Caballeriá et al., 1989, 1991; Hernández-Muñoz et al., 1990; Seitz et al., 1992). It has been hypothesized that this action decreases the gastric first-pass metabolism of ethanol, leading to increased blood alcohol levels of drinking individuals. Cimetidine is a substituted imidazole, and imidazole derivatives have long been recognized to be inhibitors of horse alcohol dehydrogenase activity (Theorell & McKinley-McKee, 1961a,b). X-ray crystallography of the horse enzyme•imidazole and enzyme•NADH•imidazole complexes indicates that imidazole coordinates directly to the active site zinc (Boiwe & Bränden, 1977; Cedergren-Zeppezauer, 1983). Steady-state kinetics of imidazole inhibition of the horse enzyme confirms that imidazole associates with free enzyme, as well as with the enzyme•NAD^+ and enzyme•NADH complexes (Theorell & McKinley-McKee, 1961a). To investigate the possible involvement of cimetidine inhibition of the human alcohol dehydrogenase isoenzymes in ethanol first-pass metabolism, the inhibition of gastric $\sigma\sigma$ and liver $\beta_2\beta_2$, $\pi\pi$, and $\chi\chi$ were examined *in vitro* at physiological pH and temperature. The inhibition of other H_2-receptor antagonists on these human alcohol dehydrogenase isoenzymes was also examined.

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[1] Abbreviations: SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DACA, 4-(dimethylamino)cinnamaldehyde. Human class I alcohol dehydrogenases include the $\alpha\alpha$, $\beta\beta$, and $\gamma\gamma$ isoenzymes, the human class II isoenzyme is $\pi\pi$, the human class III isoenzyme is $\chi\chi$, and the human gastric class IV isoenzyme is $\sigma\sigma$. Polymorphic variants of class I isoenzymes are designated by subscripts (e.g., β_2).

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MATERIALS AND METHODS

Reagents. Cimetidine (Tagamet) was supplied by Smith-Kline Beecham (Philadelphia, PA) or was obtained from Sigma; no difference in kinetics was observed from either source. Famotidine (Pepcid) and ranitidine (Zantac) were supplied by Glaxo (Cary, NC), and nizatidine (Axid) was supplied by Eli Lilly & Co. (Indianapolis, IN). Drugs were dissolved in 0.1 M sodium phosphate, pH 7.4, 37 °C, at the following maximal concentrations: 26.4 mM cimetidine (FW 252 g/mol), 72.4 mM nizatidine (FW 331 g/mol), 57.0 mM ranitidine (FW 351 g/mol), and 5.9 mM famotidine (FW 337 g/mol). Grade I NAD^+ and NADH were obtained from Sigma; NADH was obtained in preweighed vials. 4-(Dimethylamino)cinnamaldehyde[1] (DACA) was also obtained from Sigma and was dissolved in acetonitrile at a stock concentration of 5 mg/mL. All solutions were used within 4 h of preparation. Native $\sigma\sigma$ was purified from human stomach (Stone et al., 1993a). Recombinant human $\sigma\sigma$ and $\beta_2\beta_2$ expressed in *Escherichia coli* were purified as described (Kedishvili et al., 1994; Hurley et al., 1990; Stone et al., 1993b). Human $\chi\chi$ was purified from liver by the method of Wagner and coworkers (1984), with minor modifications (Yang et al., 1994). Recombinant human $\pi\pi$ was expressed in *E. coli* and purified as described (Davis et al., 1994). Protein homogeneity was evaluated by 15% SDS−PAGE,[1] starch gel electrophoresis, and isoelectric focusing (Stone et al., 1993a).

Kinetics. All kinetics were performed at 37 °C in 0.1 M sodium phosphate, pH 7.4. Inhibition kinetics with cimetidine were performed on a Gilford Response or Perkin-Elmer Lambda 6 double-beam spectrophotometer by monitoring the production or depletion of NADH at 340 nm (molar coefficient $6.22 \times 10^3\ M^{-1}\ cm^{-1}$). Cimetidine inhibition of ethanol oxidation by $\sigma\sigma$ with varied ethanol was evaluated by mixing enzyme with 2.4 mM NAD^+, six to eight ethanol concentrations (10−320 mM), and five to six cimetidine concentrations (0.2−6.4 mM). The cimetidine inhibition constant with $\beta_2\beta_2$ was performed with 0.2−12.8 mM ethanol, and the K_i value with $\pi\pi$ was performed with 2.5−320 mM ethanol. Reaction mixtures were preincubated for several minutes at 37 °C before addition of coenzyme to start the reaction.

Inhibition of ethanol oxidation with varied NAD^+ was evaluated with saturating levels of ethanol (0.2 M for $\sigma\sigma$ and 33 mM for $\beta_2\beta_2$) and with six to ten NAD^+ concentrations ranging from 4 μM to 4.5 mM. Cimetidine inhibition constants of acetaldehyde reduction were evaluated by varying either acetaldehyde or NADH. With $\beta_2\beta_2$, the inhibition constant with varied NADH was evaluated with six NADH concentrations (30−360 μM) with saturating acetaldehyde (2 mM). The inhibition constant with $\sigma\sigma$ was obtained with NADH concentrations ranging from 25 to 400 μM and with saturating acetaldehyde (150 mM). Kinetics of acetaldehyde reduction with $\sigma\sigma$ and $\beta_2\beta_2$ could not be monitored at 340 nm above 0.4 mM NADH, which is below the saturating level of NADH with the enzymes (Kedishvili et al., 1994). Cimetidine inhibition of acetaldehyde reduction by $\sigma\sigma$ and $\beta_2\beta_2$ with varied acetaldehyde was, therefore, obtained at a subsaturating NADH concentration approximating K_m^{NADH} (0.2 mM), using six to seven acetaldehyde concentrations ranging from 0.05 to 3 mM with $\beta_2\beta_2$ or from 1 to 240 mM with $\sigma\sigma$.

Initial velocities (micromoles of NADH formed or depleted per minute per micromole of enzyme) were obtained by linear regression. The amount of product formed was less than 5% within the 1−4 min during which the reaction was monitored. Acetaldehyde reducing activity with $\sigma\sigma$ was maintained below 2% product formation to avoid product inhibition. Kinetics of initial velocities were evaluated by nonlinear regression of inhibition equations using SAS (SAS Institute, Inc., Cary, NC). Analysis was performed with duplicate data points totaling 54−84. Each data set was evaluated for fit to noncompetitive, uncompetitive, and competitive inhibition models (Segel, 1975). The data were also evaluated for fit to a mixed-type inhibition, in which inhibitor binds to two different enzyme species with different affinities. In this case, the rate equation is a mixture of pure competitive and pure uncompetitive inhibition: $v = V_{max}\cdot[S]/[K_m(1 + \{[I]/K_i^c\}) + [S](1 + \{[I]/K_i^u\})]$ (Segal, 1975). The model that best fit the data was evaluated by F-statistic. Analysis with varied NADH was evaluated by setting V_{max} at 9600 and 18 000 min^{-1} with $\beta_2\beta_2$ and $\sigma\sigma$, respectively. Each data set was also evaluated for the possibility that inhibition occurs by the effective depletion of substrate, caused by a substrate·inhibitor complex (Segal, 1975). The effect of various dead-end enzyme·inhibitor complexes on inhibition kinetics was evaluated by a FORTRAN computer program based on the technique of Fisher and Schultz (1970). Values of V_{max} were calculated from a standard assay, assuming a specific activity of 92 units/mg for $\sigma\sigma$ (Kedishvili et al., 1994) 19 units/mg for $\beta_2\beta_2$ (Yin et al., 1984), and 1.7 units/mg for $\pi\pi$ (Bosron et al., 1979; Davis et al., 1994) and assuming two active sites per molecule. The standard assay conditions were 2.4 mM NAD^+ and 33 mM ethanol in 0.1 M sodium pyrophosphate, pH 8.5, for $\beta_2\beta_2$, 33 mM ethanol in 0.1 M glycine, pH 10, for $\pi\pi$, and 100 mM ethanol in 0.1 M glycine, pH 10, for $\sigma\sigma$.

The nizatidine and ranitidine samples absorbed strongly at 340 nm. Therefore, inhibition studies with these drugs and with famotidine were performed by monitoring the reduction of DACA at 398 nm. The concentration of DACA used in the reaction mixture was 60 μM. Acetaldehyde reducing activity of $\beta_2\beta_2$, $\sigma\sigma$, and $\pi\pi$ was determined with 5 mM cimetidine, famotidine, nizatidine, or ranitidine. Rates were determined at saturating and subsaturating NADH concentrations (0.2 and 2 mM NADH for $\beta_2\beta_2$ and σ-ADH or 20 μM and 0.2 mM NADH for $\pi\pi$).

Cimetidine and Nizatidine Docking to the β_2·NAD$^+$·4-Iodopyrazole Complex. Cimetidine and nizatidine binding in the active site of $\beta_2\beta_2$ was modeled with the X-ray structure of the isoenzyme complexed with NAD^+ and 4-iodopyrazole (Hurley et al., 1994). The molecules were docked into the active site with AUTODOCK (Goodsell & Olson, 1990), using a Silicon Graphics CRIMSON computer. The solvent molecules and 4-iodopyrazole were removed from the model structure, and leucine 116 was rotated 120° about the Cα−Cβ bond. This rotation was necessary to allow the substrates to enter and exit, and it is the conformation observed for leucine 116 in the human β_1·NAD^+·cyclohexanol complex. Partial charges for the protein atoms (including polar hydrogen atoms) were assigned with X-PLOR (Brünger, 1988). The active site zinc atom was assigned a charge of +2. In addition, the positive charge associated with the nicotinamide ring was distributed such that the pyridine nitrogen and C4 position of the

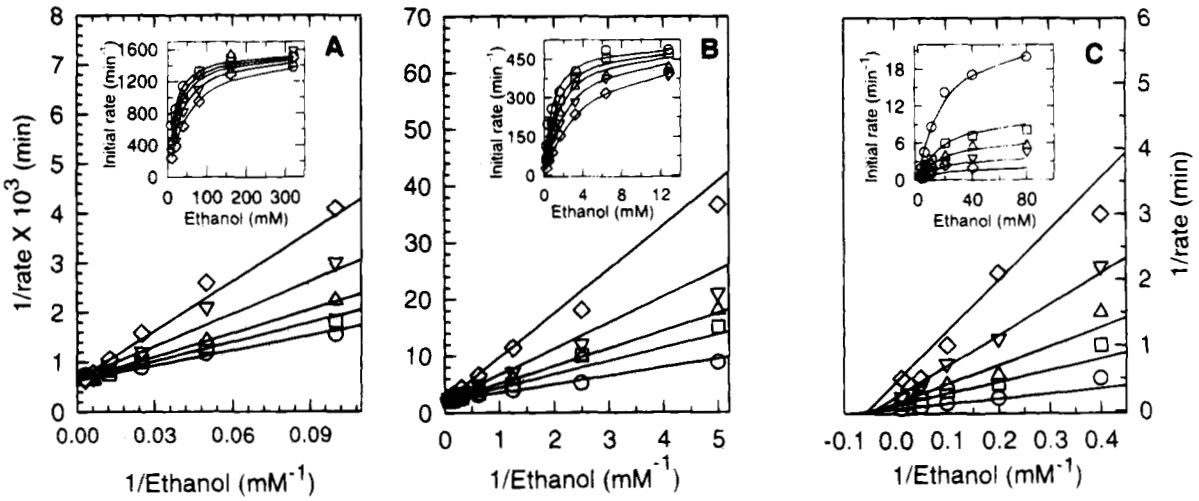


FIGURE 1: Cimetidine inhibition of gastric and liver alcohol dehydrogenase ethanol oxidizing activity. Lineweaver–Burk plots of cimetidine inhibition with varied ethanol concentrations of (A) $\sigma\sigma$, (B) $\beta_2\beta_2$, and (C) $\pi\pi$ at 2.4 mM NAD^+ are shown. Representative cimetidine concentrations are shown. Data (symbols) fit best a competitive inhibition model with (A) and (B) and noncompetitive inhibition with (C) (regression lines). Each symbol represents the average of duplicate initial rate determinations at the following cimetidine concentrations: (A) (○) 0 mM, (□) 0.8 mM, (△) 1.6 mM, (▽) 3.2 mM, and (◇) 6.4 mM; (B and C) (○) 0 mM, (□) 0.4 mM, (△) 0.8 mM, (▽) 1.6 mM, and (◇) 3.2 mM. Nonlinear regression produced the following estimates: (A) V_{max} = 1590 ± 20 min^{-1}, $K_m^{ethanol}$ = 16 ± 1 mM, and $K_i^{cimetidine}$ = 2.8 ± 0.4 mM; (B) V_{max} = 500 ± 10 min^{-1}, $K_m^{ethanol}$ = 0.77 ± 0.04 mM, and $K_i^{cimetidine}$ = 0.77 ± 0.07 mM; (C) V_{max} = 30 ± 1 min^{-1}, $K_m^{ethanol}$ = 16.3 ± 1.3 mM, and $K_i^{cimetidine}$ = 0.50 ± 0.03 mM. The insets show the Michaelis–Menten plots of the data.

pyridine ring were each assigned partial charges of +0.30, and the remaining four atoms were each assigned partial charges of +0.10. The input parameters for the program AUTODOCK were optimized for alcohol dehydrogenase by modifying the potential functions distributed with AUTODOCK. The constants associated with the carbon, nitrogen, oxygen, sulfur, and hydrogen 6-12 potentials were reduced by 5%, while a 10-12 potential was used to model the semicovalent interactions between zinc and the potential liganding atoms oxygen, nitrogen, and sulfur. The potential function constants used for the zinc atom were adjusted to allow the crystallographic observed ligand distances (~2.2 Å) between the zinc ion and its substrate or inhibitor ligands. No polar hydrogen atoms were used on the inhibitors, since productive interaction with the catalytic zinc ion is associated with proton dissociation. Therefore, the oxygen, nitrogen, and sulfur atoms in cimetidine and nizatidine were assigned partial charges of −0.5. The potential function used for the zinc ion was substituted for the C4 position of the nicotinamide ring to more closely approximate the crystallographically observed position of 4-iodopyrazole in the active site. This is due to the extremely short contact distances (2.2 Å) observed for the interaction of 4-iodopyrazole with NAD^+. This potential function for the C4 atom of NAD^+ was used throughout the docking experiments, because it had no qualitative effect on the results with other compounds.

Ten iterations of 50 cycles each were performed in the simulated annealing. Each cycle of the simulation consisted of either 50 000 or 95 000 steps. The simulation was started at a temperature of k_BT = 100 kcal/mol with a 10% decrement in temperature between each cycle. The lowest energy conformation from each cycle was used as the starting point for the next cycle, and the result from each iteration was used to initiate the next iteration. The number of steps used per cycle of simulation depended only on the rigidity of the molecule. The simulation consisted of 50 000 steps per cycle with 4-iodopyrazole and 95 000 steps per cycle with cimetidine and nizatidine.

RESULTS

With $\sigma\sigma$, cimetidine concentrations of 0.4–6.4 mM inhibited ethanol oxidation progressively (Figure 1A). Ethanol concentrations above 300 mM could substantially overcome this inhibition (Figure 1A, inset). With $\beta_2\beta_2$, ethanol concentrations above 12 mM could almost completely overcome inhibition by the drug (Figure 1B, inset). Cimetidine's action on both isoenzymes best fit competitive inhibition ($p < 0.001$ for $\sigma\sigma$ and $\beta_2\beta_2$). The cimetidine K_i values were 2.8 (±0.4) mM and 0.77 (±0.07) mM for $\sigma\sigma$ and $\beta_2\beta_2$, respectively (Table 1). Oxidation of ethanol by class I $\gamma_1\gamma_1$ and $\beta_3\beta_3$ isoenzymes with varied ethanol was also competitive with cimetidine, and K_i values were 1.3 and 0.3 mM, respectively (data not shown). In contrast, inhibition of class II $\pi\pi$ ethanol oxidation by cimetidine best fit a noncompetitive inhibition model ($p < 0.10$) with a K_i value of 0.50 (±0.03) mM (Figure 1C); increasing ethanol concentrations did not overcome cimetidine inhibition. Class III $\chi\chi$ ethanol oxidizing activity with varied ethanol was not inhibited even by 5 mM cimetidine; the enzyme is not inhibited by 4-methylpyrazole (Parés & Vallee, 1981; Wagner et al., 1984).

To further characterize cimetidine inhibition of $\sigma\sigma$ and $\beta_2\beta_2$, inhibition of ethanol oxidizing activity was determined with varied NAD^+. The data (Figures 2A and 3A) fit best competitive inhibition with $\sigma\sigma$ and $\beta_2\beta_2$ ($p < 0.01$ and $p < 0.10$, respectively). The K_i values were 5.8 (±0.9) mM for $\sigma\sigma$ and 2.6 (±0.3) mM for $\beta_2\beta_2$ (Table 1). None of the above data appeared consistent with inhibition by formation of a complex between substrate and cimetidine (data not shown).

Cimetidine inhibition of acetaldehyde reduction was also evaluated with $\sigma\sigma$ and $\beta_2\beta_2$. The data with varied NADH (Figures 2B and 3B) best fit noncompetitive inhibition ($p < 0.10$ for both enzymes). The K_i values were 20.4 (±1.8) mM and 7.5 (±0.8) mM, respectively (Table 1). Acetaldehyde reduction by $\sigma\sigma$ and $\beta_2\beta_2$ with varied acetaldehyde

Table 1: Cimetidine Inhibition Patterns with Human Alcohol Dehydrogenase Isoenzymes (mM)[a]

	ethanol oxidation		acetaldehyde reduction	
isoenzyme	varied NAD^+, satd ethanol	varied ethanol, satd NAD^+	varied NADH, satd acet	varied acet, subsatd NADH
$\beta_2\beta_2$	C ($p < 0.10$) K_i =2.6 ± 0.3	C ($p < 0.001$) K_i = 0.77 ± 0.07	N ($p < 0.10$) K_i = 7.5 ± 0.8	M ($p < 0.10$) K_i^c = 5.4 ± 1.0 K_i^u = 17.4 ± 1.9
$\sigma\sigma$	C ($p < 0.01$) K_i = 5.8 ± 0.9	C ($p < 0.001$) K_i = 2.8 ± 0.4	N ($p < 0.10$) K_i = 20.4 ± 1.8	M ($p < 0.10$) K_i^c = 1.7 ± 0.3 K_i^u = 34.7 ± 5.8

[a] The data which best fit either a competitive (C), noncompetitive (N), or mixed-type inhibition model (M) were obtained as described in Materials and Methods. Best fit was evaluated by F-statistic, and the level of significance is shown in parentheses. Cimetidine K_i estimates are listed with their associated standard errors.

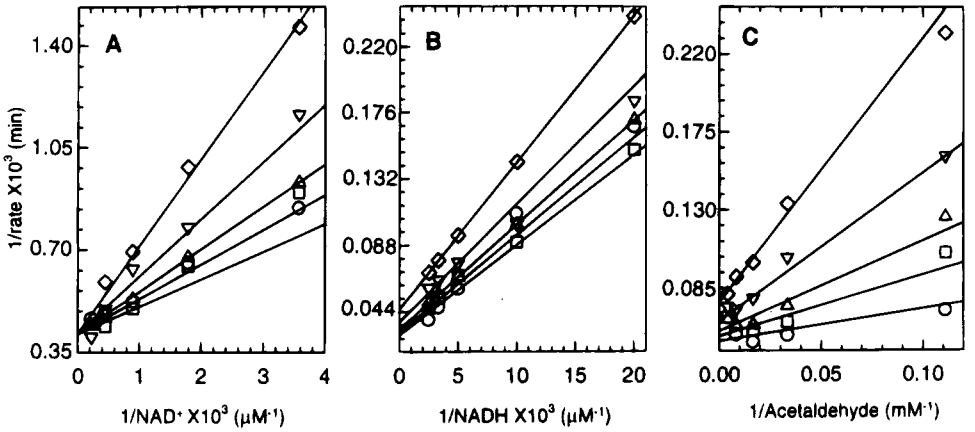


FIGURE 2: Lineweaver–Burk plots of cimetidine inhibition with human gastric $\sigma\sigma$. Cimetidine inhibition of (A) ethanol oxidation (0.2 M ethanol) with varied NAD^+, and acetaldehyde reduction with (B) varied NADH (150 mM acetaldehyde) and (C) varied acetaldehyde (0.2 mM NADH) were evaluated with $\sigma\sigma$. Symbols indicate the following representative cimetidine concentrations: (○) 0 mM, (□) 1.6 mM, (△) 3.2 mM, (▽) 6.4 mM, and (◇) 12.8 mM. (A) Data fit best a competitive inhibition model in which V_{max} = 2400 ± 20 min^{-1}, $K_m^{NAD^+}$ = 220 ± 20 μM, and $K_i^{cimetidine}$ = 5.8 ± 0.9 mM. (B) Data fit best noncompetitive inhibition in which $K_i^{cimetidine}$ = 20.4 ± 1.8 mM. (C) Data fit best a mixed-type inhibition in which V_{max} = 17 200 ± 500 min^{-1}, apparent $K_m^{acetaldehyde}$ = 3.1 ± 0.1 mM, K_i^c = 1.7 ± 0.3 mM, and K_i^u = 34.7 ± 5.8 mM.

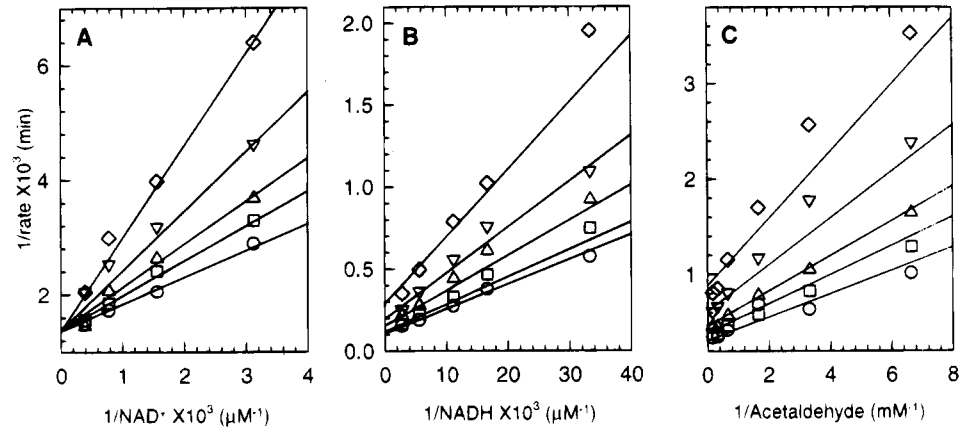


FIGURE 3: Lineweaver–Burk plots of cimetidine inhibition with human liver $\beta_2\beta_2$. Cimetidine inhibition of (A) ethanol oxidation with varied NAD^+ (33 mM ethanol), and acetaldehyde reduction with (B) varied NADH (2 mM acetaldehyde) and (C) varied acetaldehyde (0.2 mM NADH) were evaluated with $\beta_2\beta_2$. Symbols indicate the following representative cimetidine concentrations: (A and C) (○) 0 mM, (□) 0.8 mM, (△) 3.2 mM, (▽) 6.4 mM, and (◇) 12.8 mM cimetidine; (B) (○) 0 mM, (□) 3.2 mM, (△) 6.4 mM, and (▽) 12.8 mM, and (◇) 24 mM. (A) Data fit best competitive inhibition in which V_{max} = 730 ± 20 min^{-1}, $K_m^{NAD^+}$ = 340 ± 30 μM, and $K_i^{cimetidine}$ = 2.6 ± 0.3 mM. (B) Data fit best noncompetitive inhibition in which K_m^{NADH} = 142 ± 6 μM, $K_i^{cimetidine}$ = 7.5 ± 0.8 mM. (C) Data fit best a mixed-type inhibition in which V_{max} = 3400 ± 100 min^{-1}, apparent $K_m^{acetaldehyde}$ = 0.31 ± 0.02 mM, K_i^u = 17.4 ± 1.9 mM, K_i^c = 5.4 ± 1.0 mM.

(Figures 2C and 3C) was inhibited in a manner best fitting a mixed-type inhibition ($p < 0.10$ for both enzymes). The K_i values were obtained at 0.2 mM NADH, a subsaturating concentration approximating K_m^{NADH} (Table 1).

We examined whether the H_2-receptor antagonists ranitidine, nizatidine, and famotidine inhibited human alcohol dehydrogenase activity by measuring the reduction of DACA[1] (Table 2). With saturating NADH concentrations, reduction of DACA by $\beta_2\beta_2$, $\pi\pi$, $\chi\chi$, or $\sigma\sigma$ was not appreciably inhibited by 5 mM ranitidine, nizatidine, or famotidine. Under the same conditions, 5 mM cimetidine inhibited DACA reduction by $\beta_2\beta_2$, $\pi\pi$, and $\sigma\sigma$ by 36–72%, but $\chi\chi$ was not inhibited. Subsaturating NADH concentrations also showed no appreciable inhibition of DACA

Table 2: Percent Inhibition with 5 mM H_2-Receptor Antagonists[a]

	% inhibition of DACA reduction			
isoenzyme	cimetidine	ranitidine	nizatidine	famotidine
class I $\beta_2\beta_2$	36	<5	<5	0
class II $\pi\pi$	72	0	0	<5
class III $\chi\chi$	0	0	0	0
class IV $\sigma\sigma$	46	0	0	0

[a] Percent inhibition of DACA reduction with 5 mM cimetidine, ranitidine, nizatidine, or famotidine was examined with saturating levels of NADH. Percent inhibition was calculated as follows: % inhibition = [1 − (activity with drug)/(activity without drug)] × 100.

reduction by the isoenzymes with ranitidine, nizatidine, and famotidine (data not shown).

DISCUSSION

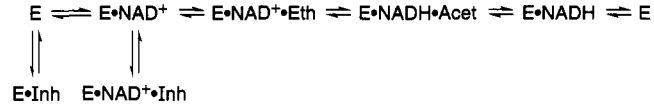
Cimetidine, an H_2-receptor antagonist used to treat peptic ulcers, is one of the most commonly prescribed drugs in the world. The drug inhibits gastric and liver class I, II, and IV alcohol dehydrogenase isoenzymes. Inhibition of ethanol oxidation with $\sigma\sigma$ and $\beta_2\beta_2$ by cimetidine is competitive with either varied ethanol or varied NAD^+. This pattern of inhibition is consistent with cimetidine associating with two enzyme species. The human class I alcohol dehydrogenase isoenzymes and gastric $\sigma\sigma$ isoenzyme function kinetically by the ordered Bi-Bi mechanism (Burnell & Bosron, 1989; Kedishvili et al., 1994). Assuming this mechansm, the two enzyme species to which cimetidine binds are the free enzyme (E) and the productive binary ($E{\cdot}NAD^+$) complex (Scheme 1, Table 3).

The cimetidine inhibition patterns of ethanol oxidation with the human $\sigma\sigma$ and $\beta_2\beta_2$ isoenzymes are identical to those observed with imidazole and cimetidine inhibition of horse alcohol dehydrogenase (Theorell & McKinley-McKee, 1961b; Carr et al., 1991). Imidazole is a metal-chelating compound that complexes directly to the active site zinc of the enzyme (Boiwe & Bränden, 1977; Cedergren-Zeppezauer, 1983). Cimetidine, which is a substituted imidazole, may also bind to the active site zinc of alcohol dehydrogenase isoenzymes.

To determine if cimetidine may complex to zinc in the active site of the human $\beta_2\beta_2$ isoenzyme in a manner analogous to that of imidazole, the drug was docked into a ternary complex ($\beta_2{\cdot}NAD^+{\cdot}$4-iodopyrazole; Hurley et al., 1994) (Figure 4). The cimetidine molecule fits well into the binding pocket, and the imidazole ring can be superimposed on the pyrazole ring in the X-ray structure. One of the cimetidine imidazole nitrogens is positioned at a distance close enough to interact with the active site zinc (about 2.8 Å). The guanidine moiety of the cimetidine molecule stretches easily along the binding pocket and makes no unfavorable interactions with the residues lining the pocket. It is, therefore, likely that cimetidine associates with zinc in the binding pocket similarly to imidazoles and pyrazoles in alcohol dehydrogenase complexes of known structure.

Access of active site zinc ligands in alcohol dehydrogenase is through either the coenzyme or substrate binding sites. The cimetidine inhibition patterns observed in this study with the human $\sigma\sigma$ and $\beta_2\beta_2$ isoenzymes are consistent with a shift in drug accessibility from the coenzyme binding site in E to the substrate binding site in $E{\cdot}NAD^+$. This shift in accessibility with cimetidine suggests that the human alcohol dehydrogenase enzyme undergoes a conformational change

Scheme 1



subsequent to NAD^+ binding. Such binary $E{\cdot}NAD^+$ and E·NADH isomerizations have been hypothesized with the horse and human alcohol dehydrogenase enzymes using kinetics and X-ray crystallography (Shore & Gutfreund, 1972; Eklund & Brändén, 1979; Geeves & Fink, 1980; Hardman, 1981; Plapp et al., 1986; Sekhar & Plapp, 1988; Stone et al., 1993b). The possibility of a binary complex isomerization is considered in Table 3; however, steady-state kinetics cannot discern if internal isomeriations are kinetically significant. Stopped-flow kinetics is required to examine the possiblity that the kinetic mechanisms of human $\sigma\sigma$ and $\beta_2\beta_2$ include a binary isomerization.

Both $\sigma\sigma$ and $\beta_2\beta_2$ are human alcohol dehydrogenase isoenzymes that exhibit high turnover numbers for ethanol oxidation (Yin et al., 1984; Moreno & Parés, 1991; Stone et al., 1993a). They are also enzymes that have a high K_m for NADH (about 0.2 mM). Cimetidine inhibition of these enzymes with varied acetaldehyde could not be evaluated with saturating NADH concentrations, because the limit of spectral detection at 340 nm is at about 0.5 mM NADH. Assuming that cimetidine inhibits according to Scheme 1, a subsaturating NADH concentration at K_m^{NADH} would produce mixed-type inhibition; this was observed with $\sigma\sigma$ and $\beta_2\beta_2$ (Table 1).

The kinetics observed here for $\sigma\sigma$ and $\beta_2\beta_2$ are consistent with cimetidine binding to the binary complex formed with NAD^+ but not to that formed with NADH. The positively charged nicotinamide moiety of bound NAD^+ may enhance the polar environment created by the active site zinc. This positive area may favor binding of the electronegative nitrogen on the imidazole moiety (Theorell & McKinley, 1961b). It is also possible that the E·NADH structure is different from that of $E{\cdot}NAD^+$, and that this difference affects cimetidine association.

The other H_2-receptor antagonists studied, nizatidine, ranitidine, and famotidine, did not inhibit DACA reduction of the alcohol dehydrogenase isoenzymes at either saturating or subsaturating levels of NADH (Table 2). DACA is an aldehyde substrate that binds only to the productive E·NADH complex of horse and human enzymes (Dunn et al., 1975; Cedergren-Zeppezauer et al., 1982; Stone et al., 1993b). When used as a substrate for a variety of alcohol dehydrogenases, it obeys the ordered Bi-Bi mechanism. Eighteen different possible mechanisms of inhibition were examined, and a sample of these mechanisms is shown in Table 3. None of these mechanisms could explain the lack of inhibition observed with both saturating and subsaturating NADH concentrations (Table 2).

The docking simulation shown in Figure 4B demonstrates that nizatidine does not fit well in the substrate binding site of β_2($NAD^+{\cdot}$4-iodopyrazole). The C2 substituent on the thiazole ring prevents orientation of the thiazole sulfur closer than 4.8 Å to the zinc atom. The compound thiazole appears to inhibit human class I alcohol dehydrogenase activity similarly to imidazole (data not shown); therefore, it appears that the substitution on the thiazole ring prevents inhibition by nizatidine and famotidine. Furan does not appear to

Table 3: Inhibition Patterns of Enzyme−Inhibitor Complexes[a]

site of inhibition									
E	(E·A ⇌ E*A)		E*AB	E*PQ	(E*Q ⇌ E·Q)		satd B, varied A	satd A, varied B	satd P, varied Q	satd Q, varied P
X							C	−	C	−
	X						U	N	U	U
		X					−	C	U	U
			X				U	U	U	U
	X					X	U	N	U	N
		X			X		U	N	U	N
			X	X			U	U	U	U
X	X						N	N	N	U
X		**X**					**C**	**C**	**N**	**U**
X			X				N	U	N	U
	X	X					U	N	U	U
X		X				X	N	U	N	U
X	X	X					N	N	N	U

[a] Predicted inhibition patterns of enzyme−inhibitor complexes were derived assuming an ordered Bi-Bi mechanism in which the binary EA and EQ complexes undergo isomerization to productive complexes (E*A and E*Q). Without a kinetically significant isomerization, EA and EQ are eliminated from the mechanism. The derivations were performed as described in Materials and Methods. Competitive (C), uncompetitive (U), and noncompetitive (N) inhibition or no inhibition (−) is predicted. With the human alcohol dehydrogenases, A = NAD^+, B = ethanol, P = acetaldehyde, and Q = NADH. The inhibition mechanism and associated inhibition pattern proposed with cimetidine inhibition of $\beta_2\beta_2$ and $\sigma\sigma$ are shown in bold.

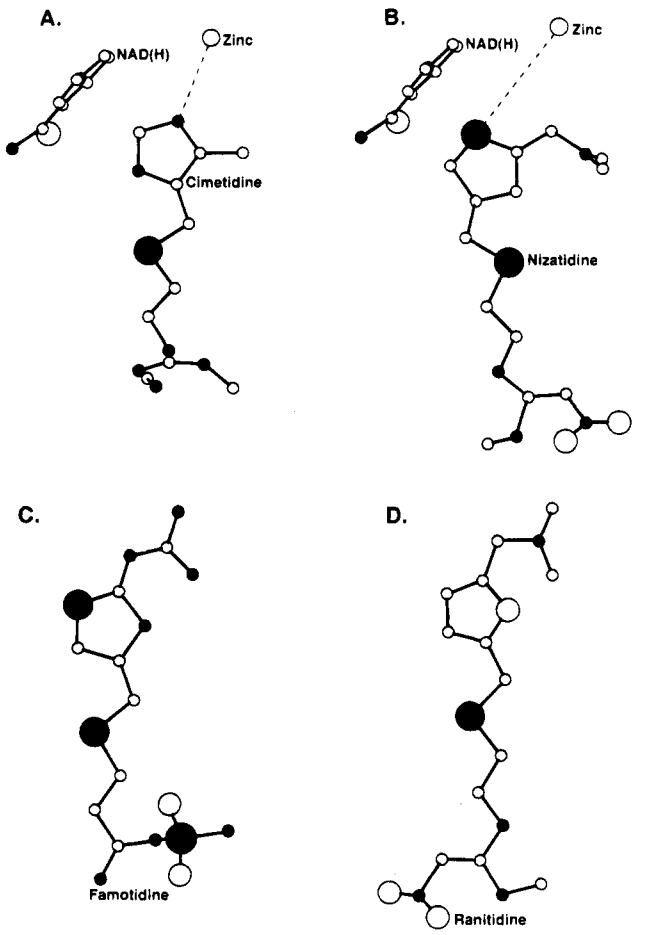


FIGURE 4: Docking of H_2-receptor antagonists into the human β_2·NAD^+·4-iodopyrazole complex. The results of docking cimetidine (A) or nizatidine (B) into the X-ray crystal structure of the human β_2·NAD^+·4-iodopyrazole complex are shown. An imidazole nitrogen of cimetidine (small filled circle) is 2.8 Å from the active site zinc (dotted line); the thiazole sulfur of nizatidine (large filled circle) is 4.8 Å from the active site zinc (dotted line). The structures of famotidine (C) and ranitidine (D) are also shown. Carbon atoms are shown as small open circles and oxygen atoms are shown as large open circles.

inhibit human class I alcohol dehydrogenase activity to a concentration of at least 100 mM (data not shown); therefore, it is not likely that ranitidine, even without the ring substitution, would inhibit alcohol dehydrogenase activity.

Omeprazole, another drug used to treat peptic ulcers, is an imidazole-containing substance with a substitution between the nitrogens on the imidazole ring. On the basis of modeling studies, we predict that the substitution on the imidazole ring would prevent the drug from coordinating to the active site zinc of alcohol dehydrogenase. Recent *in vitro* results with human stomach mucosa indicate that omeprazole does not inhibit gastric alcohol dehydrogenase activity (Roine et al., 1992; Pozzato et al., 1994).

Among H_2-receptor antagonists, cimetidine is the most widely implicated in increasing the peak blood alcohol concentrations of drinking individuals, although interpretations of clinical results are controversial [for a review see Levitt (1993); Mallat et al., 1994]. The effects of other H_2-receptor antagonists on blood alcohol concentrations are also conflicting [for a review see Levitt (1993); Burnham et al., 1994; Teyssen et al., 1994; and Toon et al., 1994]. Inhibition of ethanol oxidation by gastric and liver alcohol dehydrogenase isoenzymes during first-pass metabolism of ethanol has been postulated as the mechanism of increased blood alcohol concentrations among drinking individuals. In rats, a single dose of 20 mg/kg cimetidine could produce a gastric mucosal concentration of about 0.1−0.2 mM (Caballeriá et al., 1991). However, this is at least 10 times less than the K_i value for the drug with $\sigma\sigma$.

Human $\gamma\gamma$ and $\chi\chi$ isoenzymes are also present in stomach mucosa. Human $\gamma_1\gamma_1$ is inhibited by cimetidine at a concentration similar to the K_i value with $\sigma\sigma$, while $\chi\chi$ is not inhibited at all by the drug at 5 mM. Since the mechanism of inhibition by cimetidine is competitive with respect to ethanol, high alcohol concentrations would decrease the percent inhibition of $\gamma_1\gamma_1$ and $\sigma\sigma$ by cimetidine. Therefore, cimetidine inhibition of gastric alcohol dehydrogenase activity appears unlikely to be the cause of the observed increase in peak blood alcohol concentrations following a single treatment dose of cimetidine. Multiple treatments, however, such as a regimen of 300 mg four times daily, may produce higher amounts of cimetidine in stomach mucosa. The drugs may also affect gastric emptying rates (Levitt & Levitt, 1994; Burnham et al., 1994).

The liver is the other site of first-pass metabolism. In rats, the amount of cimetidine in the liver with a single 20 mg/kg dose can be 10-fold that in plasma (Caballeriá et al., 1991), or about 180 μM. This is at least 2-fold lower than the K_i value for cimetidine reported in this study of the human alcohol dehydrogenase isoenzymes. Activity of the liver isoenzymes with a single cimetidine dose, therefore, is probably affected very little by the drug. However, the liver is also the site of cimetidine metabolism, and the elimination half-life is about 2 h (Walkenstein et al., 1978; Shamburek & Schubert, 1993). Therefore, with multiple doses, cimetidine metabolites may accumulate; it is not known whether cimetidine metabolites inhibit alcohol dehydrogenase activity.

In summary, the kinetics of cimetidine inhibition with human class I and class IV isoenzymes are consistent with a mechanism in which the drug binds to two enzyme species: the free enzyme (E) and the productive binary ($E{\cdot}NAD^+$) complex. Cimetidine, a substituted imidazole, inhibits the human gastric and liver alcohol dehydrogenase isoenzymes at concentrations above the expected therapeutic levels of the drug. It is, therefore, unlikely that inhibition of ethanol metabolism through class I or IV alcohol dehydrogenases in either the stomach or liver accounts for the increase in blood alcohol concentrations seen with cimetidine in drinking individuals. Inhibition of alcohol dehydrogenase activity by other H_2-receptor antagonists is weak or absent.

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