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CHARACTERIZATION OF THE INDEPENDENT AND COMBINED EFFECTS OF TWO INHIBITORS ON OXIDATIVE DRUG METABOLISM IN RAT LIVER MICROSOMES

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Abstract—To evaluate how two inhibitors influence oxidative drug metabolism, this study investigated the inhibitory effects of mexiletine with cimetidine and mexiletine with lidocaine, both individually and in combination, on the oxidative metabolism of two probe substrates, aminopyrine and aniline in rat liver microsomes. Mexiletine was a competitive inhibitor of aminopyrine *N*-demethylation, whereas cimetidine was a mixed type of inhibitor ($K_i = 2.00 \pm 0.04$ and 0.20 ± 0.02 mM, respectively). For aniline hydroxylation, mexiletine exhibited a mixed type of inhibition, whereas lidocaine was a noncompetitive inhibitor ($K_i = 0.60 \pm 0.07$ and 8.50 ± 0.12 mM, respectively). The combined inhibition of either mexiletine with cimetidine or mexiletine with lidocaine on aminopyrine and aniline metabolism was close to the fully additive effects of the individual compounds when their individual concentrations were below a 2-fold K_i concentration, regardless of the apparent kinetic inhibition type. The combined inhibition was less than fully additive when the individual concentrations were twice the K_i or above. These results demonstrate that, when two inhibitors of oxidative drug metabolism are combined, both the K_i values and the concentrations of inhibitors play important roles in determining the extent of additive inhibition of enzyme activity.

Key words: cimetidine; drug metabolism; inhibition; lidocaine; mexiletine; rat liver microsomes

The P450†† enzymes are a superfamily of hemoproteins that play an essential role in the oxidative metabolism of a variety of endogenous substances and foreign compounds [1, 2]. The catalytic activities of these proteins are modulated by many factors, including genetic composition of the host [3], exposure to certain dietary and environmental chemicals [4, 5], and administration of selected medications [6]. The fact that some of the most commonly prescribed drugs are potent inhibitors of selected P450 isozymes is well documented. Previous studies from this laboratory, as well as others, have shown that mexiletine and lidocaine, class IB antiarrhythmic agents, and cimetidine, an H_2-receptor antagonist, impair the disposition of substrates that are metabolized by these isozymes [7–12]. The clinical consequences associated with these interactions are well described [13].

Despite an extensive literature on the effect of a single inhibitor on *in vivo* and *in vitro* microsomal oxidative drug metabolism, few studies have examined the response of P450 isoforms to two inhibitors administered concurrently. Loft *et al.* [14] reported that coadministration of therapeutic doses of cimetidine and disulfiram results in a partially additive inhibitory effect on antipyrine elimination in humans. In contrast, Davis *et al.* [15] showed that concurrent administration of a maximally inhibiting dose of cimetidine and a therapeutic dose of ciprofloxacin produces a further decrease in theophylline clearance compared with ciprofloxacin alone, but not with cimetidine alone. Differences in the doses of inhibitors employed, as well as the administration of fixed doses of the inhibitors to the study subjects, complicate the interpretation of these data. These factors could cause significant variation in the plasma concentrations of inhibitors within individual subjects and thus influence the outcome of the interaction. Unfortunately, there are no studies that have investigated the combined effect of different concentrations of two inhibitors on hepatic microsomal oxidative drug metabolism. Accordingly, the objectives of this *in vitro* study were to determine the inhibition kinetics of mexiletine, lidocaine, and cimetidine on the metabolism of model substrates and to characterize

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†† Abbreviations: P450, cytochrome P450; PAP, *p*-aminophenol; G-6-P, glucose-6-phosphate; and G-6-PD, glucose-6-phosphate dehydrogenase.

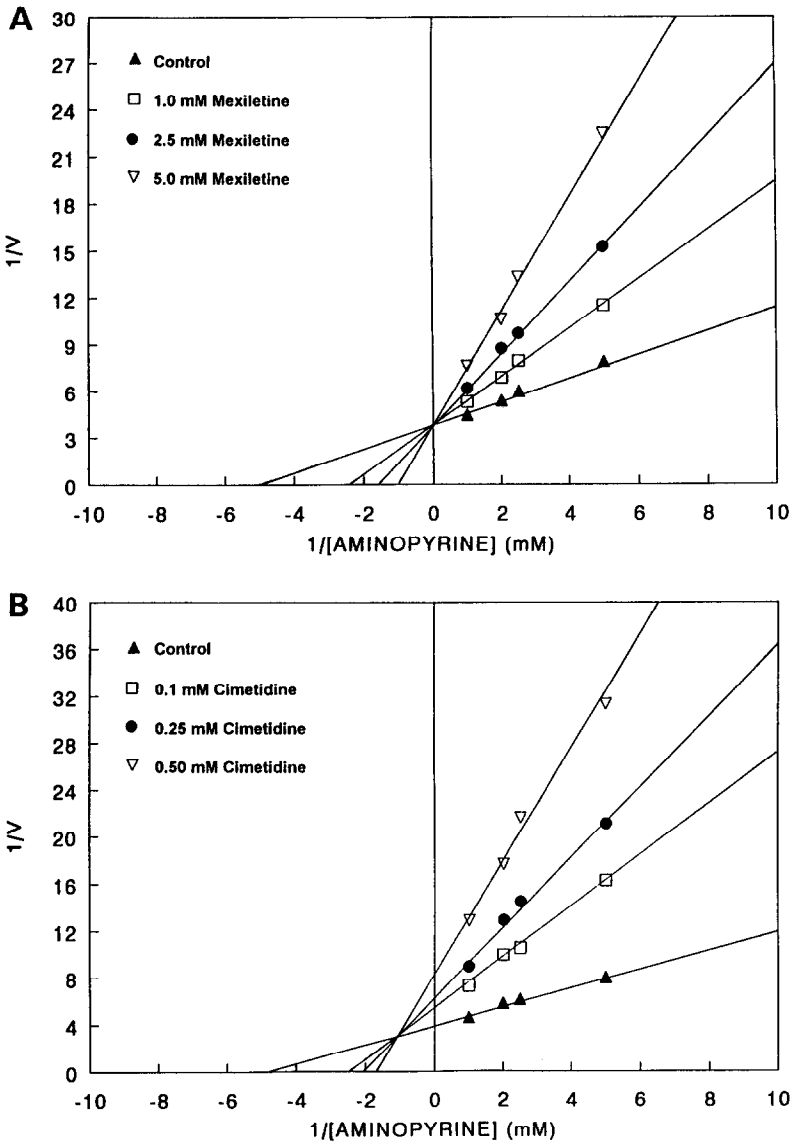

Fig. 1. Lineweaver–Burk plots of aminopyrine N-demethylation in the presence or absence of mexiletine (A) or cimetidine (B) at various concentrations. Each data point is the mean of three measurements. The plots are representative of at least four different experiments.

the individual and combined inhibitory effects of various concentrations of these compounds on hepatic drug metabolism.

MATERIALS AND METHODS

Chemicals. Mexiletine hydrochloride was provided by Boehringer-Ingelheim (Ridgefield, CT). Cimetidine, lidocaine, NADP, NADPH, aminopyrine, aniline, PAP, magnesium chloride, G-6-P, G-6-PD, BSA, formaldehyde, DMSO, glycerol, sodium phosphate, and potassium phosphate were purchased from the Sigma Chemical Co. (St. Louis, MO).

Animals. Adult male Sprague–Dawley rats weighing 250–300 g (obtained from Bantin & Kingman Co., Freemont, CA) were used for this study. The animals were housed four per cage under standard conditions of 12-hr light/dark cycles with free access to food and water.

Preparation of microsomes. The rats were euthanized by decapitation, and the livers were homogenized in ice-cold 0.05 M sodium phosphate buffer (pH 7.4). Liver microsomes were prepared by differential ultracentrifugation [16] and were stored in 0.05 M phosphate buffer (pH 7.4) with 0.1 mM EDTA and 20% glycerol at −70° until used. Microsomal protein content was determined by the method of Lowry *et al.* [17] using BSA as the standard. Total microsomal P450 content was estimated by the method of Omura and Sato [18].

Table 1. Aminopyrine N-demethylation

	K_m (mM)	V_{max} (nmol/mg protein/min)
Control	0.220 ± 0.013	0.276 ± 0.016
Mexiletine		
1.0 mM	0.510 ± 0.070*†	0.298 ± 0.028
2.5 mM	0.700 ± 0.078*	0.312 ± 0.033
5.0 mM	0.980 ± 0.023*†	0.266 ± 0.066
Cimetidine		
0.1 mM	0.360 ± 0.026*†	0.175 ± 0.009*†
0.25 mM	0.440 ± 0.020*	0.160 ± 0.005*
0.5 mM	0.530 ± 0.040*†	0.126 ± 0.005*†

Data are expressed as means ± SEM, N = 4. Each experiment was conducted in triplicate.

* Significantly different compared with control by Dunnett's test, $P < 0.05$.

† Significantly different for multiple comparisons by Tukey's test, $P < 0.05$.

Microsomal incubation. Mixtures containing 2 mg of microsomal protein and an NADPH-generating system consisting of 1 mM NADP, 10 mM G-6-P, 5 units of G-6-PD, and 5 mM magnesium chloride in 0.05 M sodium phosphate buffer (pH 7.4) were used for all incubations. All reactions were initiated by the addition of the NADPH-generating system and were conducted at 37° in an oscillating water bath. The linearity of reaction velocities with respect to time and protein concentration was confirmed prior to all experiments.

Aminopyrine N-demethylase assay. The rate of aminopyrine N-demethylation was estimated from the formation of formaldehyde. Aminopyrine (0.25 to 2.0 mM) and semicarbizide (5 mM) were added to the incubation mixtures to a final volume of 1 mL. Reactions were terminated after 15 min by the addition of 0.4 mL of 15% zinc sulfate and 0.4 mL of saturated barium hydroxide. The precipitated protein was removed by centrifugation, and the concentration of formaldehyde was quantified by the method of Nash [19] and was compared with a standard curve prepared from commercially available formaldehyde.

Aniline hydroxylase assay. The hydroxylation of aniline to PAP was determined as previously described [20]. Aniline (0.1 to 0.5 mM) was added to the incubation mixture to a final volume of 1 mL. Reactions were terminated after 20 min by the addition of 200 μL of 20% trichloroacetic acid, and the precipitated proteins were removed by centrifugation. The resultant supernatant was mixed with 0.5 mL of 10% sodium carbonate and 1 mL of 1% phenol in 0.1 M sodium hydroxide for measurement of PAP.

In vitro *kinetic and inhibition studies.* Mexiletine (1–5 mM), lidocaine (1–5 mM), or cimetidine (0.1–0.5 mM) was added to the standard incubation mixture for 3 min prior to initiation of the reaction. The V_{max} and K_m of the reactions were determined from Lineweaver–Burk double-reciprocal plots, and the K_i values were derived from Dixon plots. Inhibition models were determined using the ENZYME program [21]. Individual and combined effects of these compounds on aminopyrine and aniline metabolism were determined by preincubating these inhibitors with the microsomal protein for 3 min prior to initiating the reaction. The concentrations of the three inhibitors ranged from 0.5 to 4 times their respective K_i values. The substrate concentrations were maintained at 1 mM, and the reaction conditions were as described above.

Statistical analysis. Four experiments were performed in triplicate for each assay condition. The data are expressed as means ± SEM. Percent inhibition of aminopyrine and aniline metabolism by these compounds individually and in combination was compared using one-way analysis of variance and either Dunnett's test or Tukey's test for comparison with control or for multiple comparisons, respectively, with $P < 0.05$ considered significant.

RESULTS

Lineweaver–Burk plots demonstrated that mexiletine is a competitive inhibitor of aminopyrine N-demethylation and that cimetidine exhibits a mixed type of inhibition. In a representative experiment, pretreatment with 1.0, 2.5, and 5.0 mM mexiletine did not alter the V_{max} of aminopyrine metabolism, whereas the K_m increased from a control value of 0.20 mM to 0.41, 0.60, and 1.00 mM, respectively (Fig. 1A). Preincubating microsomes with 0.1, 0.25, and 0.5 mM cimetidine altered both K_m and V_{max} of aminopyrine N-demethylation with K_m increasing from 0.21 mM to 0.40, 0.47, and 0.60 mM, respectively, and with V_{max} decreasing from 0.26 to 0.18, 0.16, and 0.12 nmol/min/mg protein (Fig. 1B). The K_m and V_{max} values for all experiments (N = 4) are summarized in Table 1. Dixon plots (not shown) revealed that the K_i values for the inhibition of aminopyrine metabolism by mexiletine and cimetidine were 2.00 ± 0.04 and 0.20 ± 0.02 mM, respectively.

The *in vitro* kinetics of the inhibition of aniline metabolism by mexiletine and lidocaine from a representative experiment are shown in Fig. 2. Mexiletine was a mixed inhibitor of aniline hydroxylation. Following pretreatment with 1.0, 2.5, and 5.0 mM mexiletine, the V_{max} for PAP formation

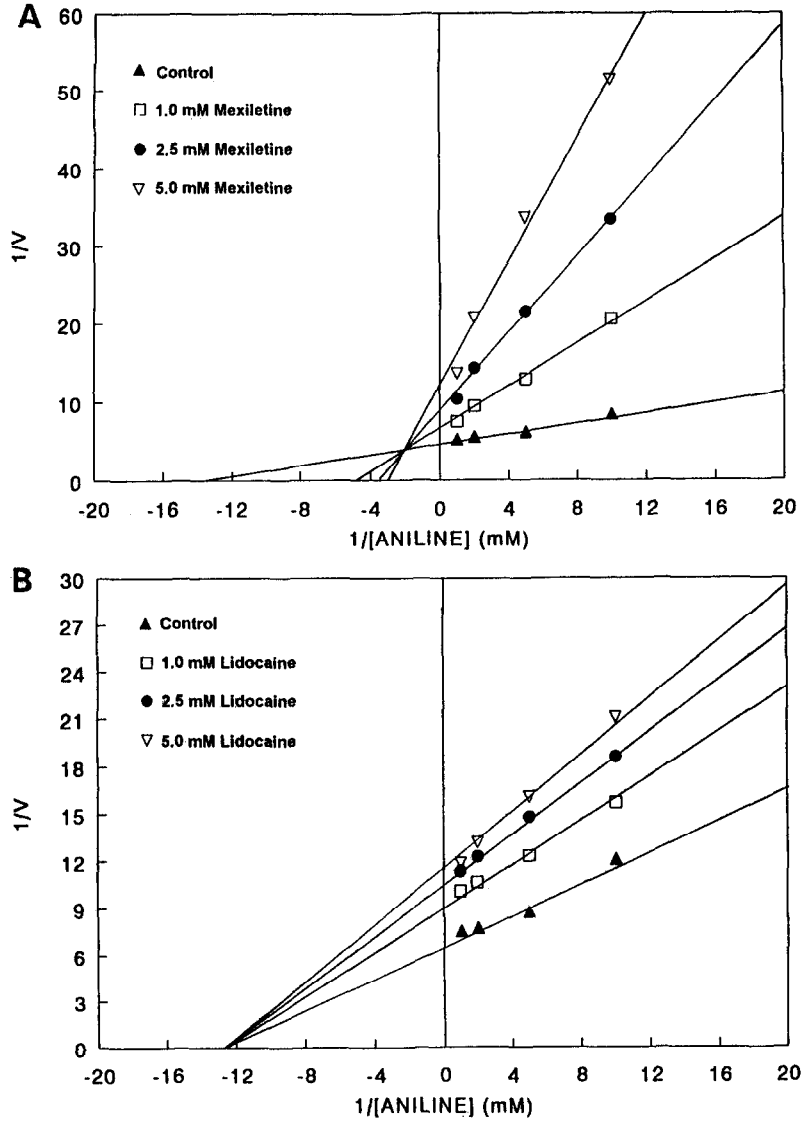

Fig. 2. Lineweaver–Burk plots of aniline hydroxylation in the presence or absence of mexiletine (A) and lidocaine (B) at various concentrations. Each data point is the mean of three measurements. The plots are representative of at least four different experiments.

Table 2. Aniline hydroxylation

	K_m (mM)	V_{max} (nmol/mg protein/min)
Control	0.076 ± 0.007	0.190 ± 0.011
Mexiletine		
1.0 mM	0.238 ± 0.024*†	0.140 ± 0.005*†
2.5 mM	0.314 ± 0.031*	0.110 ± 0.005*
5.0 mM	0.397 ± 0.043*†	0.082 ± 0.002*†
Lidocaine		
1.0 mM	0.071 ± 0.002	0.124 ± 0.005*†
2.5 mM	0.072 ± 0.003	0.109 ± 0.004*
5.0 mM	0.074 ± 0.003	0.100 ± 0.004*†

Data are expressed as means ± SEM, N = 4. Each experiment was conducted in triplicate.

* Significantly different compared with control by Dunnett's test, $P < 0.05$.

† Significantly different for multiple comparisons by Tukey's test, $P < 0.05$.

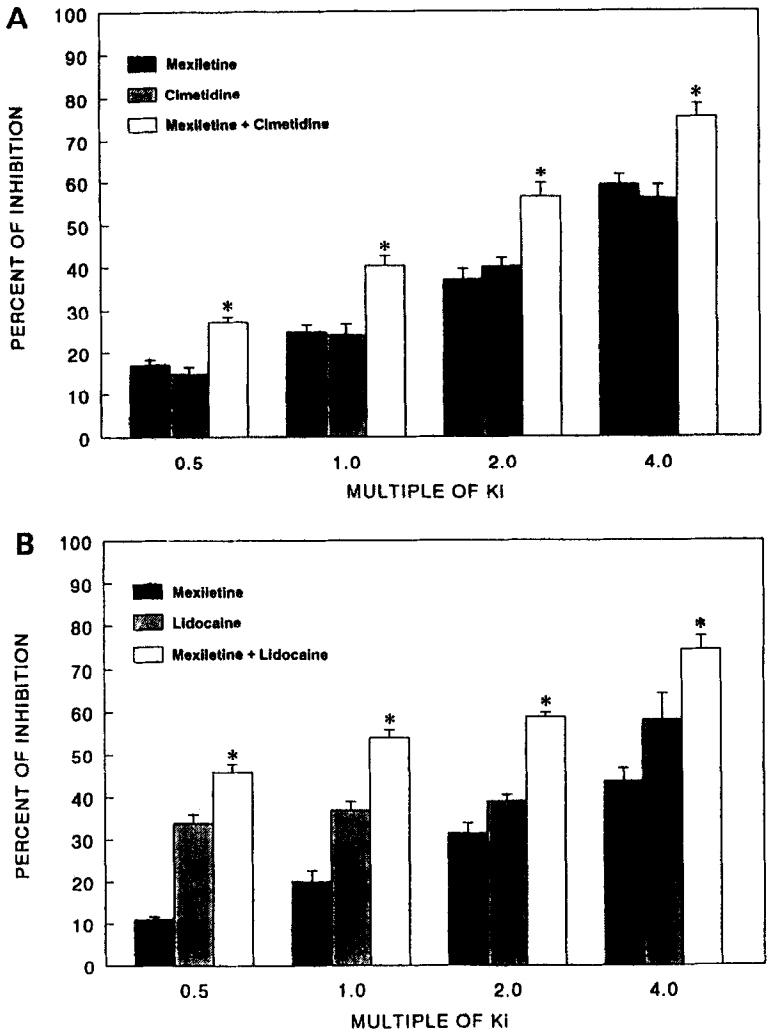

Fig. 3. Individual and combined inhibitory effects of mexiletine and cimetidine on aminopyrine N-demethylation (A) and mexiletine and lidocaine on aniline hydroxylation (B). Data are expressed as the means ± SEM of six experiments. Key: (*) combined inhibitory effect was greater than either individual inhibitory effect ($P < 0.05$).

decreased from a control value of 0.22 to 0.15, 0.11, and 0.08 nmol/min/mg protein, respectively, whereas the K_m increased from a control value of 73 μM to 200, 270, and 330 μM (Fig. 2A). In contrast, lidocaine was a noncompetitive inhibitor of aniline hydroxylation. Preincubation with 1.0, 2.5, and 5.0 mM lidocaine decreased the V_{max} from a control value of 0.16 and 0.11, 0.097, and 0.089 nmol/min/mg protein, respectively, whereas K_m did not change (Fig. 2B). Table 2 summarized the values of K_m and V_{max} for all experiments. The K_i values derived from Dixon plots (not shown) for inhibition of aniline metabolism by mexiletine and lidocaine were 0.60 ± 0.07 and 8.50 ± 0.12 mM, respectively.

The individual and combined inhibitory effects of mexiletine and cimetidine on aminopyrine N-demethylation and mexiletine and lidocaine on aniline hydroxylation are shown in Fig. 3. Within the concentration ranges used in the present study, pretreatment with two compounds in combination impaired the oxidative metabolism of aminopyrine and aniline to a greater extent than either inhibitor alone ($P < 0.05$, all comparisons). The combined inhibition of either mexiletine with cimetidine or mexiletine with lidocaine on aminopyrine and aniline metabolism was close to the fully additive effects of the individual agents when their individual concentrations were below twice their respective K_i values. Even though the inhibition did not reach maximal or saturated conditions, the combined inhibition was less than fully additive when the individual inhibitor concentrations were twice their K_i values or above.

DISCUSSION

Aminopyrine and aniline were used as model substrates for this study. Although drug metabolism may involve a single or multiple P450 isoforms, the

metabolism of most drugs is mediated by multiple enzymes. In the rat, for example, aminopyrine N-demethylation is mediated by multiple P450 isoforms, including CYP1A1, 1A2, 2B1, 2B2, 2C11, and 2C12 [22]. However, aniline hydroxylation is mediated predominantly by CYP2E1 [23]. Therefore, these two substrate reactions afford two different model systems in which to study drug metabolism *in vitro*.

Mexiletine and lidocaine are class IB antiarrhythmics, which are used commonly in the treatment of ventricular arrhythmias. Clinical studies indicate that mexiletine decreased the clearance of caffeine and theophylline by 40–50% [10, 11, 24], suggesting that mexiletine may inhibit cytochrome P450 isozymes. Our previous *in vitro* study demonstrated that the class IB antiarrhythmic compounds mexiletine, lidocaine and tocainide are inhibitors of the CYP1A family in 3-methylcholanthrene-induced rat liver microsomes [12]. Our present data confirm that mexiletine impairs the *in vitro* oxidative metabolism of aminopyrine and aniline in a concentration-dependent manner. The apparent kinetics of inhibition revealed that mexiletine is a competitive inhibitor of aminopyrine demethylation and a mixed inhibitor of aniline hydroxylation, whereas lidocaine is a noncompetitive inhibitor of aniline hydroxylation. The inhibitory potency of mexiletine on aniline hydroxylation was much greater than that of lidocaine. The K_i of mexiletine was 0.6 mM, a value more than 14-fold lower than that of lidocaine (8.5 mM).

Cimetidine, a commonly prescribed H_2-receptor antagonist, inhibits the activity of P450 isozymes by binding to the heme portion of the enzyme [25, 26]. *In vitro* and *in vivo* studies have shown that cimetidine impairs the biotransformation of various substrates of the P450 enzyme system [7–9, 13]. Preincubating hepatic microsomes with cimetidine decreased aminopyrine N-demethylation (K_i = 0.2 mM) to a much greater extent than aniline hydroxylation (K_i = 6.1 mM). An analysis of the apparent kinetics of inhibition demonstrated that cimetidine is a mixed inhibitor of aminopyrine *N*-demethylation, but a noncompetitive inhibitor of aniline hydroxylation. Cimetidine is insoluble in buffer at concentrations above 5 mM unless DMSO is used as a solvent. Because cimetidine is a weak inhibitor for aniline hydroxylation and the concentrations used in the combined inhibition study were based on the multiplicity of their K_i values, the higher concentrations of cimetidine required a volume of DMSO that overwhelmingly inhibited the incubation system. Thus, lidocaine was chosen as the second inhibitor for the experiments in which aniline was the substrate.

Inhibition of P450 isozymes is a common mechanism responsible for drug–drug interactions. Despite numerous studies of this subject, there is only limited information concerning the effect of combinations of two inhibitors on hepatic oxidative drug metabolism, and the results of these investigations are divergent [14, 15, 27]. The most likely explanation for the discrepancy is that different doses of inhibitors were used. This suggests that the dose (or concentration) of the inhibitors present in the system is an important determinant of the response to combined inhibition. The present study tested this hypothesis in an *in vitro* system using K_i-related inhibitor concentrations. A wide range of inhibitor concentrations can be used to characterize the inhibitory response to two different inhibitors in an *in vitro* system. Our data demonstrated that the effect of combined inhibition by two inhibitors, mexiletine with cimetidine and mexiletine with lidocaine, was close to the fully additive effect of the two inhibitors when the individual concentrations were below twice their respective K_i values. The combined inhibition was less than fully additive when the individual concentrations were equal to or greater than a 2-fold K_i value. This concentration-dependent response to the combined inhibitors was not influenced by the apparent kinetics of the interaction (competitive inhibition vs mixed inhibition) among the substrates and inhibitors. These *in vitro* data are consistent with the hypothesis that the additivity of the response to two inhibitors is K_i and concentration dependent. These data are also consistent with those from *in vivo* studies [15, 27].

In summary, this study demonstrated that since mexiletine impairs *in vitro* aminopyrine N-demethylation and aniline hydroxylation, this drug is an inhibitor of multiple P450 isoforms. Both mexiletine and lidocaine inhibited aniline metabolism, but mexiletine was approximately 14-fold more potent than lidocaine as an inhibitor of aniline hydroxylation. Exposure of these substrates to two inhibitors simultaneously caused additive or partially additive inhibition. The extent of inhibition was determined by the concentrations and K_i values of the individual inhibitors in the system, regardless of the apparent kinetic inhibition type of each inhibitor and whether single or multiple isoforms were involved.

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