

Chloramphenicol Is a Potent Inhibitor of Cytochrome P450 Isoforms CYP2C19 and CYP3A4 in Human Liver Microsomes

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The inhibitory effect of chloramphenicol on human cytochrome P450 (CYP) isoforms was evaluated with human liver microsomes and cDNA-expressed CYPs. Chloramphenicol had a potent inhibitory effect on CYP2C19-catalyzed *S*-mephenytoin 4'-hydroxylation and CYP3A4-catalyzed midazolam 1-hydroxylation, with apparent 50% inhibitory concentrations (inhibitory constant [K_i] values are shown in parentheses) of 32.0 (7.7) and 48.1 (10.6) μ M, respectively. Chloramphenicol also weakly inhibited CYP2D6, with an apparent 50% inhibitory concentration (K_i) of 375.9 (75.8) μ M. The mechanism of the drug interaction reported between chloramphenicol and phenytoin, which results in the elevation of plasma phenytoin concentrations, is clinically assumed to result from the inhibition of CYP2C9 by chloramphenicol. However, using human liver microsomes and cDNA-expressed CYPs, we showed this interaction arises from the inhibition of CYP2C19- not CYP2C9-catalyzed phenytoin metabolism. In conclusion, inhibition of CYP2C19 and CYP3A4 is the probable mechanism by which chloramphenicol decreases the clearance of coadministered drugs, which manifests as a drug interaction with chloramphenicol.

Chloramphenicol, a broad-spectrum antibiotic, has been used to treat severe infections for several decades. Its use in contemporary medical practice has fallen out of favor due to dose-dependent and idiosyncratic bone marrow suppression (9). However, chloramphenicol showed good in vitro activity against many vancomycin-resistant enterococcal infections and is useful for certain life-threatening infections (22, 30).

Chloramphenicol increased the plasma drug concentrations or effects of several drugs, such as cyclosporine, tacrolimus, and phenytoin (4, 10, 14, 17, 20, 25, 29, 32, 36, 38).

Cytochrome P450s (CYPs) play a key role in the oxidation of numerous endogenous and exogenous compounds (24, 38). In previous animal studies, in vivo treatment with chloramphenicol has been shown to inhibit CYP-catalyzed reactions in microsomal preparations in rats, suggesting that the drug interactions of chloramphenicol possibly involve the CYPs (2, 8, 15).

However, there are no published studies investigating the effect of chloramphenicol on different CYP isoforms in human liver microsomes. Therefore, we studied the inhibitory effect of chloramphenicol on the activities of the major CYP isoforms in human liver microsomes and cDNA-expressed CYPs, using selective marker reactions to clarify the mechanism underlying the drug-drug interactions of chloramphenicol.

MATERIALS AND METHODS

Materials. Chloramphenicol, phenytoin, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH), phenacetin, acetaminophen, chlorzoxazone, paclitaxel, dextromethorphan, dextrorphan, furafylline, NADP, EDTA, $MgCl_2$, glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase (G-6-PDH) were purchased from Sigma-Aldrich (St. Louis, Mo.). 1-Hydroxymidazolam, *S*-warfarin, 7-hydroxywarfarin, *S*-mephenytoin, 6-hydroxypaclitaxel, and 4'-hydroxymephenytoin were ob-

tained from Ultrafine Chemical Co. (Manchester, United Kingdom). Acetonitrile and methanol were acquired from Fisher Scientific Co. (Pittsburgh, Pa.). Midazolam was kindly provided by Bukwang Pharmaceutical Co. (Seoul, Korea). All other reagents and chemicals used were of analytical or high-performance liquid chromatography (HPLC) grade. Human liver microsomes containing specific content of CYPs from donors (HG-43, HG-56, and HG-89) were obtained from Gentest Corp. (Woburn, Mass.). Human cDNA-expressed recombinant CYPs (1A2, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) were also purchased from Gentest Corp.

Enzyme assay studies. All incubations were performed in duplicate, and the mean values were used. We measured the activities of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 by using specific reaction probe drugs. The K_m and V_{max} values were determined in human liver microsomes for the CYP isoform-specific reactions used, for which the incubation conditions and method references are listed in Table 1.

We used here the free base form of chloramphenicol, which is microbiologically active. A 25 mM chloramphenicol stock solution was prepared by dissolving chloramphenicol in distilled water, and this solution was serially diluted in distilled water. The reaction metabolite of the probe drug for each CYP was quantified by interpolating the peak area ratios of the respective metabolite and the internal standard from a standard curve of known metabolite concentrations.

Each incubation was performed with cDNA-expressed CYPs or human liver microsomes in a final incubation volume of 0.25 ml, after they were diluted from their original stock concentrations (final concentrations, 0.25 to 1 mg/ml for microsomes and 20 to 40 pmol/liter for cDNA-expressed CYPs). The incubation medium contained 100 mM phosphate buffer (pH 7.4) containing an NADPH-generating system (including 1.3 mM NADP, 3.3 mM G-6-P, 3.3 mM $MgCl_2$, and 1.0 U of G-6-PDH per ml). For each substrate (probe drug), preliminary experiments were performed to determine whether metabolite formation was linear with respect to incubation time and microsomal protein concentrations. After incubation at 37°C with the probe drugs and microsomes in the absence (control) and presence of the NADPH-generating system, reactions were stopped by placing the reactants on ice and adding the appropriate stop solution (described in the corresponding reference in Table 1).

CYP2C19-catalyzed *S*-mephenytoin 4'-hydroxylation was a modification of the reaction described by Ko et al. (13). After *S*-mephenytoin (25 to 100 μ M) was incubated with human liver microsomes (1 mg/ml) for 30 min at 37°C in the presence of the NADPH-generating system, the reaction was stopped by placing it on ice and adding 70 μ l of perchloric acid. Carbamazepine was added as the internal standard. The mixture was centrifuged at 10,000 \times g for 5 min at 4°C, and aliquots of the supernatant were injected onto an HPLC system (Lichrosorb RP18 [4.6 by 250 mm]; UV, 225 nm; 320:680 acetonitrile–1% acetic acid [pH 4.0] at 0.7 ml/min).

CYP3A4-catalyzed midazolam 1-hydroxylation was performed by a modifica-

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TABLE 1. CYP isoform-specific probe drug reactions

CYP	Index reaction	Final microsomal concn (mg/ml)	Incubation time (min)	K_m (μM) ^a	V_{\max} (pmol/min/mg protein) ^b	Reference
CYP1A2	Phenacetin <i>O</i> -deethylation	2	25	35	879	27
CYP2C9	<i>S</i> -Warfarin 7-hydroxylation	0.25	15	9.7	14.5	41
CYP2C19	<i>S</i> -Mephenytoin 4'-hydroxylation	1	30	87	295	13
CYP2D6	Dextromethorphan <i>O</i> -demethylation	0.5	30	11	255	1
CYP2C8	Paclitaxel 6 α -hydroxylation	1	20	25	400	40
CYP3A4	Midazolam 1-hydroxylation	0.25	5	12	550	23
CYP2E1	Chlorzoxazone 6-hydroxylation	0.4	25	85	960	19

^a V_{\max} , the maximum velocity of metabolite formation.

^b K_m , Michaelis-Menten constant.

tion of the method of Perloff et al. (23). After microsomes (0.25 mg/ml) were incubated for 30 min with midazolam (5 to 50 μM), the reaction was stopped with acetonitrile. Ketoconazole was added as the internal standard. Aliquots of the supernatant were injected onto an HPLC system for analysis (Capcell Pak C₁₈ [1.5 by 250 mm]; UV, 245 nm; 265:45:690 acetonitrile-methanol-0.4 N acetate buffer [pH 4.0] at 0.12 ml/min).

CYP isoform-specific reactions for CYP1A2 (27), CYP2C8 (40), CYP2C9 (41), CYP2E1 (19), and CYP2D6 (1) are described in the corresponding references listed in Table 1.

To determine whether the inhibition of CYP isoform(s) by chloramphenicol is mechanism based, chloramphenicol was preincubated with the incubation medium at 37°C for 0 to 15 min, in either the presence or absence of the NADPH-generating system. After preincubation, the probe substrates were added either with or without the NADPH-generating system.

The HPLC system consisted of a Shiseido 2001 pump (Shiseido Co., Tokyo, Japan), a Shiseido model 2023 autosampler (Shiseido Co.), a dsChrom2000 integrator (Donam Instruments, Suwon, Korea), a Shiseido 2002 UV detector (Shiseido Co.), and a Jasco FP-2020 Plus fluorescence detector (Jasco Co., Tokyo, Japan).

Data analysis. Fifty percent inhibitory concentrations (IC_{50} s [concentration of the inhibitor required to cause 50% inhibition of the original enzyme activity]) were determined graphically by using curves of mean enzyme activity versus inhibitor concentrations. The apparent inhibitory constant (K_i) values were calculated by graphical analysis from secondary plots of slopes taken from double-reciprocal plots of chloramphenicol concentrations versus metabolite formation.

RESULTS

Inhibitory effect of chloramphenicol on CYP-catalyzed reactions. Chloramphenicol strongly inhibited CYP2C19-catalyzed *S*-mephenytoin 4'-hydroxylation and CYP3A4-catalyzed midazolam 1-hydroxylation in human liver microsomes, with apparent IC_{50} s of 32.0 and 48.1 μM , respectively (Fig. 1). The Lineweaver-Burk plot, Dixon plots, and secondary reciprocal plots indicated that chloramphenicol inhibited CYP2C19 activity with a mixed-type inhibition pattern, with an apparent K_i value of 7.7 μM (Fig. 2 and Table 2). In the case of the CYP3A4-catalyzed reaction, chloramphenicol exhibited competitive inhibition with an apparent K_i value of 10.6 μM (Fig. 3 and Table 2). Chloramphenicol also showed competitive and moderate inhibition of the CYP2D6-catalyzed reaction with an IC_{50} (K_i) of 375.9 (75.8) μM , but did not inhibit or only minimally inhibited the other CYP-catalyzed reactions tested (Fig. 1). To evaluate whether chloramphenicol acts as a mechanism-based inhibitor of CYP-catalyzed reactions, we preincubated chloramphenicol with NADPH for 15 min prior to the addition of the CYP-specific substrates, but this did not increase the degree of inhibition (data not shown).

The mechanism of chloramphenicol-induced inhibition of phenytoin metabolism. The inhibitory effect of chloramphenicol

predominantly affected CYP2C19 and CYP3A4, but affected CYP2D6 only moderately. Therefore, we investigated the mechanism by which chloramphenicol interacts with phenytoin, which is metabolized mainly by CYP2C9 and to a lesser extent by CYP2C19 (3, 7, 35), leading to an increase in phenytoin levels in reported cases of the drug interaction. Chloramphenicol showed a weak inhibitory effect on phenytoin *p*-hydroxylation to HPPH, with an apparent IC_{50} of 619.7 μM (Fig. 4). Using cDNA-expressed CYPs, we confirmed that CYP2C9 and CYP2C19 resulted in HPPH metabolite formation at velocities of 29.5 ± 3.4 and 31.2 ± 5.2 pmol/min/pmol of CYP, respectively. However, little or no HPPH metabolite was formed from the other cDNA-expressed CYPs tested. When the inhibitory effect of chloramphenicol on CYP2C9- and CYP2C19-catalyzed phenytoin *p*-hydroxylation was evaluated, HPPH formation catalyzed by CYP2C9 was not inhibited, but the CYP2C19-catalyzed reaction was strongly and dose-dependently inhibited by chloramphenicol, suggesting

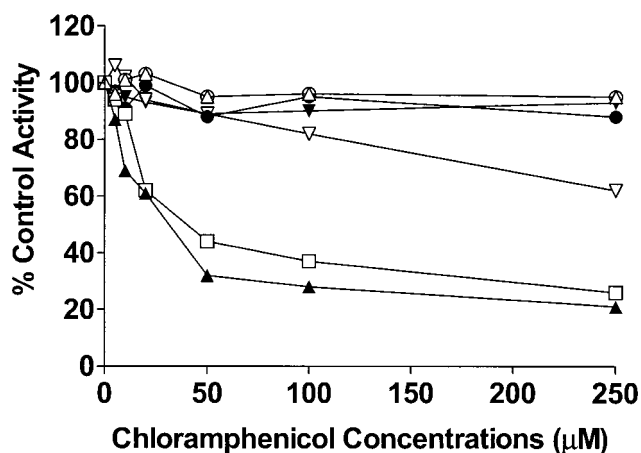


FIG. 1. Inhibitory effect of chloramphenicol on CYP-catalyzed reactions in human liver microsomes. Effects of chloramphenicol (0 to 250 μM) on CYP1A2-catalyzed phenacetin *O*-deethylation (○), CYP2C8-catalyzed paclitaxel 6 α -hydroxylation (●), CYP2C9-catalyzed *S*-warfarin 7-hydroxylation (Δ), CYP2C19-catalyzed *S*-mephenytoin 4'-hydroxylation (\blacktriangle), CYP2D6-catalyzed dextromethorphan *O*-demethylation (∇), CYP2E1-catalyzed chlorzoxazone 6-hydroxylation (\blacktriangledown), and CYP3A4-catalyzed midazolam 1-hydroxylation (\square). Each data point indicates the average value calculated from three different liver microsomal preparations.

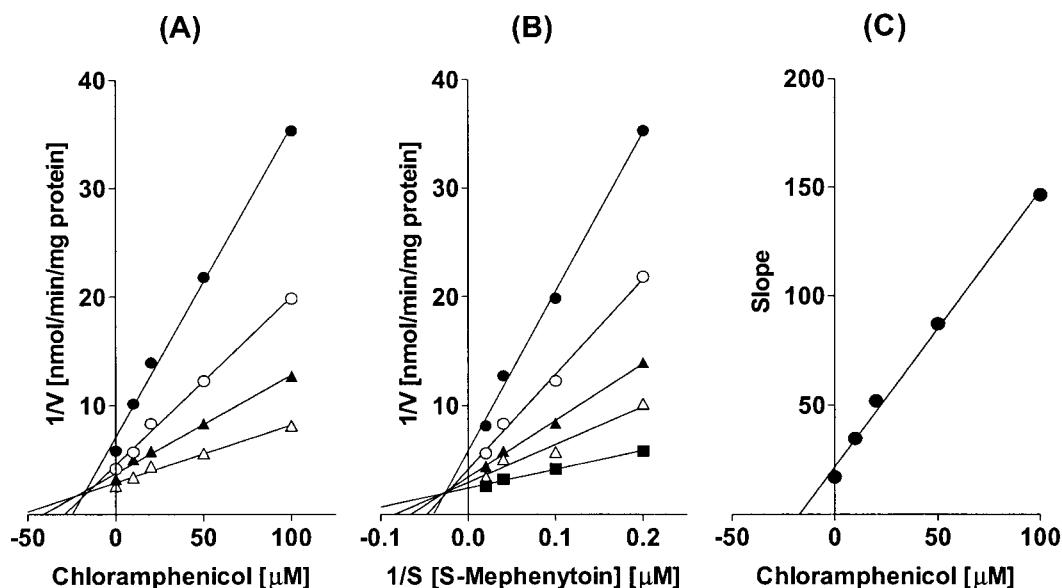


FIG. 2. (A) Representative Dixon plot for the inhibition by chloramphenicol (0 to 100 μM) of CYP2C19-catalyzed *S*-mephenytoin 4'-hydroxylation in human liver microsomes with 25 (\bullet), 50 (\circ), 75 (\blacktriangle), or 100 (\triangle) μM *S*-mephenytoin. (B) Lineweaver-Burk plot of CYP2C19-catalyzed *S*-mephenytoin 4'-hydroxylation in the absence (\blacksquare) or presence of 10 (\triangle), 20 (\blacktriangle), 50 (\circ), or 100 (\bullet) μM chloramphenicol. (C) Secondary plot of slopes taken from Lineweaver-Burk plots versus chloramphenicol concentration. Each data point represents the average of duplicate measurements.

that the drug interaction between phenytoin and chloramphenicol involves the inhibition of CYP2C19 (Fig. 4).

DISCUSSION

Studies using rats or mice have shown that chloramphenicol is an inhibitor of CYP in liver microsomes (2, 24, 26). It has therefore been presumed a priori that chloramphenicol is an inhibitor of CYPs in humans. Furthermore, several drug interactions between chloramphenicol and coadministered drugs have supported this hypothesis (4, 10, 14, 17, 20, 25, 29, 32, 36, 38). However, little has been known of the inhibitory effect of chloramphenicol on human CYPs until now. In the present study, we confirmed that chloramphenicol has an inhibitory effect on CYP2C19, CYP3A4, and, to a lesser extent, CYP2D6 in human liver microsomes.

Chloramphenicol has been suggested to be effective in the treatment of vancomycin-resistant enterococcal infections in transplantation patients (22, 30). Calcineurin inhibitors, including cyclosporine and tacrolimus, interact with chloramphenicol when used in a combination therapy, causing an increase in their plasma drug concentrations and/or their toxicity (4, 20, 32, 36). Because both tacrolimus and cyclosporine are metabolized via CYP3A4 (16, 31, 37), the results of the present study indicate that the inhibition of CYP3A4-catalyzed reactions by chloramphenicol is the underlying mechanism by which the concentrations of both cyclosporine and tacrolimus in plasma are elevated by the administration of chloramphenicol.

In contrast, chloramphenicol is purported to be a CYP2C9 inhibitor, because chloramphenicol interacts with several drugs, such as phenytoin, warfarin, and phenobarbital (10, 14, 17, 25, 28, 38), which are mainly metabolized via CYP2C9 (7, 18, 21,

41). Interestingly, we observed no inhibitory effect of chloramphenicol on CYP2C9 in human liver microsomal preparations.

The CYP2C subfamily is the second-most-abundant CYP protein in the human liver following CYP3A (30%), representing about 20% of the total CYPs (34). CYP2C9 is clearly the most abundant CYP2C protein expressed, followed by CYP2C19 and CYP2C8 (28). Among the CYP2C isoforms tested, chloramphenicol only inhibited CYP2C19-catalyzed reaction. Phenytoin is metabolized by CYP2C9 and, to a lesser extent, by CYP2C19 (3, 7, 18, 35). Although CYP2C9 is mainly responsible for phenytoin metabolism, several drugs, such as tricyclic antidepressants, ticlopidine, omeprazole, and cimetidine, also influence concentrations of phenytoin in plasma through the inhibition of CYP2C19, but not through CYP2C9 inhibition (5, 6, 11). In the present study, chloramphenicol selectively inhibited CYP2C19-catalyzed reaction and not

TABLE 2. Inhibition of CYP2C19, CYP2D6, and CYP3A4 isoforms by chloramphenicol and predicted *in vivo* inhibition of the metabolism of coadministered CYP2C19, CYP2D6, and CYP3A4 substrates by chloramphenicol, respectively, from *in vitro* data

CYP isoform	IC_{50} (K_i) (μM) ^a	Type of inhibition	Predicted <i>in vivo</i> inhibition [$I/(I+K_i)$] ^b
CYP2C19	32.0 (7.7)	Mixed ($\alpha = 4.2$)	0.62–0.80
CYP2D6	375.9 (75.8)	Competitive	0.14–0.29
CYP3A4	48.1 (10.6)	Competitive	0.54–0.74

^a K_i values are derived from secondary plots of slopes taken from double-reciprocal plots versus chloramphenicol concentrations, and the IC_{50} s were calculated graphically.

^b The inhibitor concentration (I) of chloramphenicol (therapeutic concentrations, 10 to 25 $\mu\text{g}/\text{ml}$ [31 to 77 μM]) and the fraction of drug unbound to protein are about 40% (see Discussion for details).

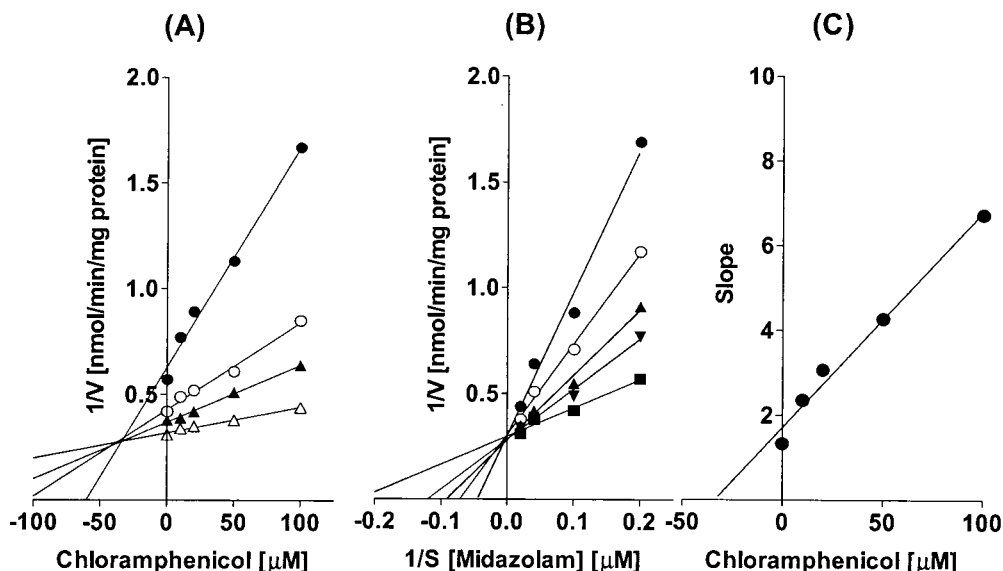


FIG. 3. (A) Representative Dixon plot for the inhibition by chloramphenicol (0 to 100 μ M) of CYP3A4-catalyzed midazolam 1-hydroxylation in human liver microsomes with 5 (●), 10 (○), 25 (▲), or 50 (△) μ M midazolam. (B) Lineweaver-Burk plot of CYP3A4-catalyzed midazolam 1-hydroxylation in the absence (■) or presence of 10 (△), 20 (▲), 50 (○), or 100 (●) μ M chloramphenicol. (C) Secondary plot of slopes taken from Lineweaver-Burk plots versus chloramphenicol concentration. Each data point represents the average of duplicate measurements.

CYP2C9 in microsomal preparations. Furthermore, only CYP2C19-catalyzed phenytoin *p*-hydroxylation was inhibited by chloramphenicol. Taken together, these results indicate that chloramphenicol is an inhibitor of CYP2C19, and not of CYP2C9.

We found several reports of drug interactions between chloramphenicol and warfarin, but chloramphenicol did not inhibit *S*-warfarin 7-hydroxylation catalyzed by CYP2C9 in the present study. However, we cannot rule out the possibility that another isoform of warfarin (*R*-warfarin) interacts with chloramphenicol, because warfarin is a chiral drug composed of *S* and *R* forms (11). *S*-Warfarin is metabolized by CYP2C9, whereas *R*-warfarin is metabolized mainly by CYP1A2 and CYP3A4 (7, 11). Moreover, when we incubated a racemate of warfarin with chloramphenicol in human liver microsomes, we found chloramphenicol inhibited *R*-warfarin metabolism, whereas *S*-warfarin was not inhibited (data not shown). We have also found numerous case reports of interactions between warfarin and CYP3A4 inhibitors (12, 33, 42). Taken together,

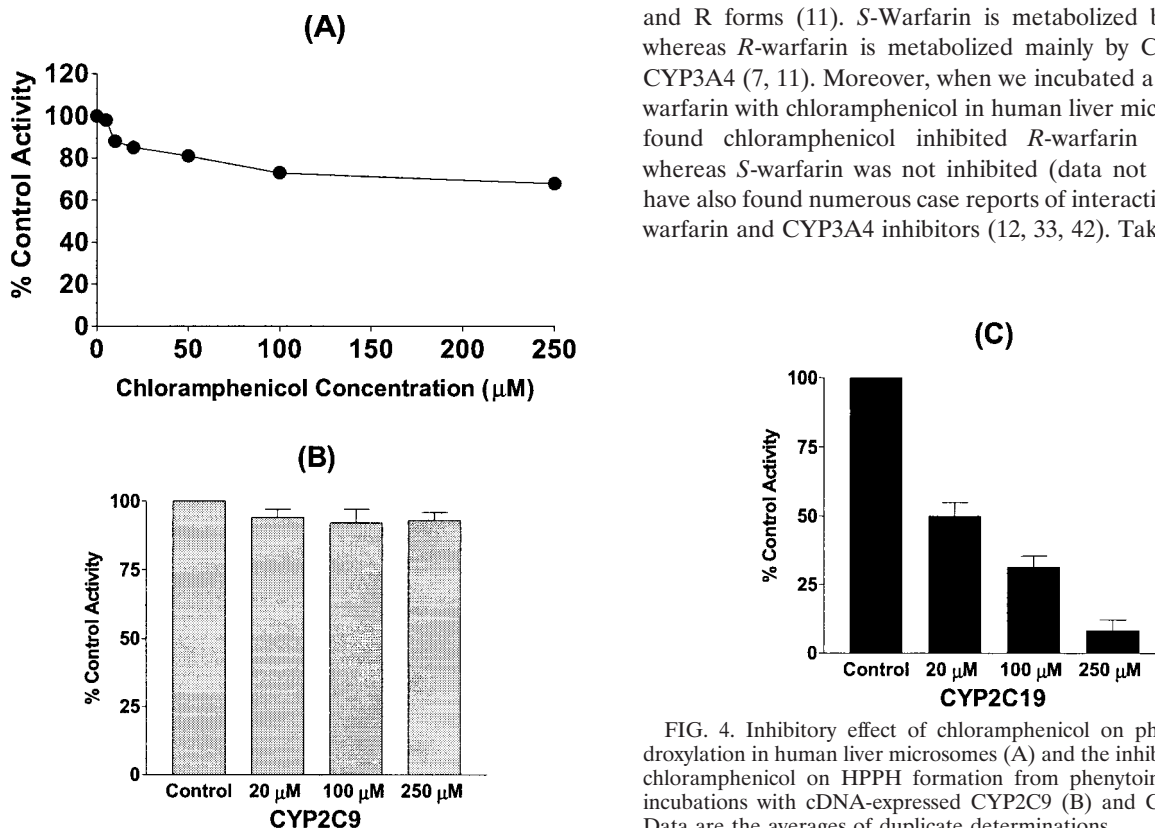


FIG. 4. Inhibitory effect of chloramphenicol on phenytoin *p*-hydroxylation in human liver microsomes (A) and the inhibitory effect of chloramphenicol on HPPH formation from phenytoin (25 μ M) in incubations with cDNA-expressed CYP2C9 (B) and CYP2C19 (C). Data are the averages of duplicate determinations.

these data suggest that CYP3A4 inhibition by chloramphenicol may be the mechanism underlying the inhibition of warfarin metabolism, especially the inhibition of *R*-warfarin.

Theoretically, drug interactions based on the inhibition of hepatic metabolism, i , can be predicted by both the K_i value and the concentration of the inhibitor, $[I]$, around the metabolic enzyme in the liver by using the following predictive model: $i = [I]/([I] + K_i)$, assuming that the substrate concentration is much lower than its K_m value (39). Based on the hypothesis that only the unbound forms of a drug in plasma are available for diffusion to intrahepatic regions of metabolic activity, the unbound concentration of an inhibitor in plasma has been used to predict *in vivo* drug interactions. Considering that therapeutic concentrations of chloramphenicol in plasma are 31 to 77 $\mu\text{mol/liter}$ and protein binding is around 60%, we would expect approximately 54 to 80% inhibition of the clearance of CYP3A4 and CYP2C19 substrates by chloramphenicol (Table 2), suggesting that chloramphenicol is a potent inhibitor of CYP3A4 and CYP2C19 *in vivo*. Even though CYP2D6 was not potentially inhibited by chloramphenicol, it also showed a 14 to 29% inhibition of clearance, suggesting that circumstances such as hypoalbuminemia and elevated concentrations of chloramphenicol and environmental factors (i.e., genetic polymorphism of CYP2D6), may affect the clearance of coadministered CYP2D6 substrates.

In conclusion, our *in vitro* study demonstrates that chloramphenicol at clinically relevant concentrations has a potent inhibitory effect on CYP2C19, CYP3A4, and, to a lesser extent, CYP2D6. The inhibition of CYP2C19 and CYP3A4 by chloramphenicol may explain the mechanism of the interactions between chloramphenicol and coadministered drugs.

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