Short Communication

MUTUAL INHIBITION BETWEEN QUININE AND ETOPOSIDE BY HUMAN LIVER MICROSOMES

Evidence for Cytochrome P4503A4 Involvement in Their Major Metabolic Pathways

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ABSTRACT:
The mutual inhibition between quinine and etoposide with their major metabolic pathways (i.e., quinine 3-hydroxylation and etoposide 3′-demethylation) was examined in vitro by human liver microsomes. Etoposide inhibited quinine 3-hydroxylation in a concentration-dependent manner with a mean IC50 of 65 μM. The mean maximum inhibition by etoposide (100 μM) of quinine 3-hydroxylation was about 60%. Similarly, etoposide 3′-demethylation was inhibited by quinine in a concentration-related manner with a mean IC50 value of 90 μM. The mean maximum inhibition by quinine (100 M) of etoposide 3′-demethylation was about 52%. An excellent correlation (r = 0.947, p < 0.01) between quinine 3-hydroxylase and etoposide 3′-demethylase activities in six different human liver microsomes was observed. Two inhibitors of CYP3A4, ketoconazole (1 μM) and troleandomycin (100 μM), inhibited quinine 3-hydroxylation by about 90% and 80%, and etoposide 3′-demethylation by about 75% and 65%, respectively. We conclude that quinine and etoposide mutually inhibit the metabolism of each other, consistent with the previous finding that CYP3A4 catalyzes the metabolism of both substrates.

Quinine is recommended for the treatment of chloroquine-resistant Plasmodium falciparum malaria and is an important drug of choice for the treatment of complicated and/or cerebral malaria (Tracy and Webster, 1996; Hien et al., 1996; Boele van Hensbroek et al., 1996). However, its most common use outside of countries with endemic malaria is as treatment for leg cramps (Dyer et al., 1996). The most widely used antimalarial drug, the detailed metabolism of which has been recently elucidated: the formation of 3-hydroxyquinine from quinine in mouse, rat, and dog liver microsomes. Etoposide inhibited quinine 3-hydroxylation in a concentration-dependent manner with a mean IC50 of 65 μM. The mean maximum inhibition by etoposide (100 μM) of quinine 3-hydroxylation was about 60%. Similarly, etoposide 3′-demethylation was inhibited by quinine in a concentration-related manner with a mean IC50 value of 90 μM. The mean maximum inhibition by quinine (100 M) of etoposide 3′-demethylation was about 52%. An excellent correlation (r = 0.947, p < 0.01) between quinine 3-hydroxylase and etoposide 3′-demethylase activities in six different human liver microsomes was observed. Two inhibitors of CYP3A4, ketoconazole (1 μM) and troleandomycin (100 μM), inhibited quinine 3-hydroxylation by about 90% and 80%, and etoposide 3′-demethylation by about 75% and 65%, respectively. We conclude that quinine and etoposide mutually inhibit the metabolism of each other, consistent with the previous finding that CYP3A4 catalyzes the metabolism of both substrates.

Materials and Methods

Drugs and Chemicals. Synthetic 3-hydroxyquinine was a generous gift from Dr. P. Winstanley (University of Liverpool, Liverpool, UK). Quinine, ketoconazole, and troleandomycin (TAO) were purchased from Sigma Chemical Co. (St. Louis, MO). Etoposide, 3′-demethyletoposide and the internal standard (4′-demethyllepidodendoryltoxin-9-(4,6-D-propylidene-B-D-glucopyranoside) were obtained from Nippon Kayaku Co., Ltd. (Tokyo, Japan). Acetonitrile, methanol, and other reagents of analytical grade were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). NADP+, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan).

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Preparation of Human Liver Microsomes. Six histologically normal liver samples were obtained from Japanese patients with hepatic metastatic cancer (from colon or rectum) who underwent a partial hepatectomy at the Division of General Surgery, International Medical Center of Japan, Tokyo, as excess material that was removed during surgery on the liver as reported previously (Chiba et al., 1993; Echizen et al., 1993). Ethical approval for the study had been granted by the Institutional Ethics Committee of the International Medical Center of Japan. Washed microsomes were prepared by classical differential centrifugation technique (Chiba et al., 1993; Echizen et al., 1993). After the determination of microsomal protein by the method of Lowry et al. (Lowry et al., 1951), aliquots of the individual microsomal samples were stored at −80°C until used.

Assay with Human Liver Microsomes. The basic incubation medium contained 0.1 mg/ml human liver microsomes, 0.5 mM NADP⁺, 2.0 mM glucose-6-phosphate dehydrogenase, 4 mM MgCl₂, 0.1 mM EDTA, 100 mM potassium phosphate buffer (pH 7.4), and 100 μM of quinine or 50 μM of etoposide in a final volume of 250 μl. All the reactions, except for TAO, were initiated by the addition of the NADPH-generating system without preincubation, and the mixture was incubated at 37°C in a shaking water bath for 15 min. After the reaction was stopped by the addition of 500 μl ice-cold methanol for quinine metabolism or 100 μl ice-cold acetonitrile for etoposide metabolism, 100 μl of 1M sodium-phosphate buffer (pH 3.0) as well as 50 μl of 5 μM internal standard was added to the etoposide metabolism mixture. The mixture was centrifuged at 10,000g for 10 min and the supernatant was injected onto a high-performance liquid chromatography (HPLC) system as described below.

3-Hydroxyquinine was measured in the incubation mixture by HPLC using fluorometric detection, according to a published method (Wanwimolruk et al., 1996) with minor modifications as employed in our recent study (Zhao et al., 1993). The inter- and intra-assay coefficients of variation for each procedure (N=6) were <10%, and the lowest limits of detection for both 3-hydroxyquinine and quinine, defined as the lowest concentration with a signal-to-noise ratio of 10, were 5 ng/ml.

The determination of 3'-demethylotoposide was performed by HPLC with fluorescence detection. The HPLC system consisted of a model L-7100 pump (Hitachi Ltd., Tokyo, Japan), a model L-7480 fluorescence detector (Hitachi), a model L-7200 autosampler (Hitachi), a model D-7500 integrator (Hitachi), and a 4.6 × 75 mm Develosil ODS-HG-3 column (Nomura Chemical Co., Ltd., Aichi, Japan). The mobile phase consisted of acetonitrile-potassium dihydrogenphosphate (20 mM) in a proportion of 24/76 (v/v), and was delivered at a flow rate of 0.8 ml/min. The column temperature was maintained at 25°C by a model SM-05 water circulator (Taisei, Tokyo, Japan). The eluate was monitored at the excitation and emission wavelengths of 288 nm and 328 nm, respectively, by using the fluorescence detector as mentioned above. Sixty milliliters of sample was injected onto the HPLC system. The intra- and inter-assay coefficients of variation were < 5.0% and < 3.0%, respectively.

The detection limit of 3'-demethylotoposide was 25 pmol/tube.

Inhibition Study. The drugs tested for a possible inhibitory effect on quinine 3-hydroxylation or etoposide 3'-demethylation were dissolved in methanol. To identify the respective IC₅₀ (50% inhibition of quinine 3-hydroxylation or etoposide 3'-demethylation compared with the respective control values), various test drug concentrations, ranging from 0.001 to 100 μM, were chosen. Quinine and etoposide concentrations were set at 100 μM and 400 μM, respectively, according to the respective apparent kinetic parameters for quinine 3-hydroxylation and etoposide 3'-demethylation.

Correlation Study. Different concentrations of quinine, ranging from 25–400 μM, and those of etoposide, ranging from 5–125 μM, were used in the experiments for assessing the respective apparent kinetic parameters for quinine 3-hydroxylation and etoposide 3'-demethylation in six different human liver microsomes. The apparent kinetic parameters (i.e. apparent Kₘ and Vₘₜₐₓ for etoposide) were estimated according to the Eadie-Hofstee equation by use of the nonlinear least-squares regression analysis program, MULTI (Yamaoka et al., 1981). Then, the apparent intrinsic clearance for the two substrates was estimated as 

\[ \text{IC}_{50} = \frac{V_{\text{max}}}{K_m} \]

Statistical Analysis. All values are expressed as means ± SD. Correlation between the quinine 3-hydroxylation and etoposide 3'-demethylation activities was assessed from the apparent Vₘₜₐₓ/Kₘ values as described above.

Results and Discussion

Chromatograms and Assessment of Incubation Conditions. With the described chromatographic conditions, no interfering peaks for 3-hydroxyquinine, 3'-demethylotoposide, or the internal standard were present in the incubation mixture. The formation of both 3-hydroxyquinine from quinine and 3'-demethylotoposide from etoposide were time-, NADPH- and microsome-dependent, suggesting the possible involvement of P450(s) in their metabolism. P450 involvement was later confirmed by the inhibition study using specific inhibitors of CYPs as well as by recombinant studies for quinine (Zhao et al., 1996) and etoposide (Kawashiro et al., unpublished data) metabolism. Preliminary studies revealed that the 3-hydroxylation of quinine with human liver microsomes was linear with regard to the incubation time from 5 to 60 min when 100 μM (around Kₘ value in human liver microsomes) of quinine was incubated with microsomes equivalent to 0.1 mg of protein/ml. A linear relationship was also observed between the rate of the metabolite production for up to 15 min and protein concentration for up to 0.25 mg/ml. On the other hand, the formation rate of 3'-demethylotoposide was linear at 37°C for up to 15 min when 50 μM (around Kₘ value in human liver microsomes) of etoposide and 0.1 mg/ml microsomal protein were present. A linear relationship was also observed between the rate of metabolite production at 15 min and protein concentration for up to 0.2 mg/ml. Accordingly, the subsequent inhibition studies were performed with a 15-min incubation and a microsomal protein content of 0.1 mg/ml for both quinine 3-hydroxylation and etoposide 3'-demethylation.

Mutual Inhibition between Quinine and Etoposide. The effect of
etoposide on the 3-hydroxylation of quinine is shown in Fig. 1. Etoposide inhibited the microsomal metabolism of quinine 3-hydroxylation in a concentration-dependent manner by human liver microsomes with a mean $IC_{50}$ value of $65 \mu M$ (Fig. 1). The mean maximum inhibition produced by etoposide (100 $\mu M$) on the 3-hydroxylation of quinine was about 60% compared with the control values, indicating that etoposide is a weak inhibitor of quinine 3-hydroxylation in vitro.

The effect of quinine on the 3'-demethylation of etoposide is shown in Fig. 1. The results showed that the etoposide 3'-demethylation was also inhibited by quinine in a concentration-related manner in human liver microsomes with a mean $IC_{50}$ value of 90 $\mu M$. The mean maximum inhibition produced by quinine (100 $\mu M$) on the 3'-demethylation of etoposide was about 52% compared with the control values, indicating that quinine is also a weak inhibitor of the etoposide 3'-demethylation in human liver microsomes. Although the therapeutic plasma concentrations of quinine and etoposide are about 8–60 $\mu M$ (2.5–20 $\mu g/ml$) (White, 1988; White, 1992) and up to 80 $\mu M$ (50 $\mu g/ml$) (Hande et al., 1984; Watkins et al., 1995; Maurice et al., 1995), thus, the concentrations of etoposide and quinine used in this study were relevant to clinical practice.

The possibility of drug interaction between quinine and etoposide in vivo remains to be investigated further. This is because an in vivo drug interaction should be dependent on blood or, more importantly, hepatic tissue drug concentrations. Moreover, several other factors including protein binding are excluded from an in vitro experiment.

**Correlation Study.** Fig. 3 shows an excellent correlation ($r = 0.947, p < 0.01$) between the quinine 3-hydroxylase and etoposide 3'-demethylase activities in six different human liver microsomes, indicating that the respective metabolic pathways of quinine and etoposide were catalyzed mainly by one same human CYP isoform. However, the linear regression line of the formation rate of 3-hydroxyquinine vs 3'-demethyltoeposide gave a small $x$-intercept (when $y = 0$), suggesting the possibility that other minor enzyme(s) may also be involved in the metabolism of both the drugs tested herein in human liver microsomes. This result is in a good agreement with our previous findings that both CYP3A4 (major) and CYP2C19 (minor) are involved in quinine 3-hydroxylation in human liver microsomes (Zhao et al., 1996). In addition, our previous study showed that CYP3A4 is the main, but not the sole, isoenzyme involved in etoposide 3'-demethylation. Several other minor CYP isoforms also seem to catalyze this pathway in human liver microsomes (Kawashiro et al., unpublished data).

**Inhibition Study by Ketoconazole and TAO.** To confirm further that CYP3A4 is a major isoenzyme involved in quinine 3-hydroxylation and etoposide 3'-demethylation, we employed two well-known CYP3A4 inhibitors, ketoconazole and TAO (Newton et al., 1995; Watkins et al., 1985), to perform an inhibition study in human liver microsomes. Although ketoconazole was regarded as a general inhibitor of several CYP subfamilies (e.g., CYP3A4, CYP2C9, and CYP2C19) (Glue and Banfield, 1996), studies have shown its specificity toward CYP3A4 when present at a low concentration (<2 $\mu M$) (Newton et al., 1995; Maurice et al., 1995). Thus, the concentrations of ketoconazole used in this study were <2 $\mu M$ (i.e., 0.1 and 1.0 $\mu M$). The effects of co-incubation with the inhibitors on quinine 3-hydroxylation and etoposide 3'-demethylation are shown in Fig. 4. The plots showed that both quinine 3-hydroxylation and etoposide 3'-demethylation were markedly inhibited by ketoconazole or TAO. However, the magnitude of inhibition differed between the data derived from the mutual inhibition (a maximal inhibition of 60%) and chemical inhibition experiments presented here (a maximal inhibition of 90%). Although we do not know the exact reason(s) for this observation, the possibility cannot be ignored that their respective binding constants to the protein may differ, and therefore a different extent of the mutual vs chemical inhibition might have been observed. Furthermore, the different inhibitory capacities can also be explained in this case because quinine and etoposide are not solely metabolized by CYP3A4. Indeed, other minor CYP isoforms like CYP2C19 for quinine metabolism (Zhang et al., 1996) and CYP1A2 and 2E1 for etoposide metabolism (Kawashiro et al., unpublished data) are also involved in their major metabolic pathways. Nevertheless, these inhibition results by ketoconazole and TAO provided further strong evidence that CYP3A4 is the main isoform involved in both quinine 3-hydroxylation and etoposide 3'-demethylation. This is in accordance with the published results (Zhao et al., 1996; Zhang et al., 1997; Zhao and Ishizaki, in press; Relling et al., 1992).

In conclusion, the present in vitro data with human liver microsomes suggest that quinine and etoposide each inhibit the other’s metabolism and CYP3A4 predominantly catalyzes the metabolism of both substrates in humans. However, whether the mutual inhibition between quinine and etoposide observed in this in vitro study may occur in vivo requires further investigations in patients.
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