Short Communication

MUTUAL INHIBITION BETWEEN QUININE AND ETOPOSIDE BY HUMAN LIVER MICROSOMES

Evidence for Cytochrome P4503A4 Involvement in Their Major Metabolic Pathways

(Received August 7, 1997; accepted November 11, 1997)

This paper is available online at http://www.dmd.org

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 μM) 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., 1997). It is known that quinine has a relatively low therapeutic index with some adverse reactions such as cinchonism, hypoglycemia, and cardiac arrhythmias (White, 1988). Thus, quinine-drug interactions seem to be of clinical importance.

The primary route of the systemic elimination of quinine in humans is known to be via extensive hepatic metabolism with less than 20% of the drug excreted unchanged in urine (Tracy and Webster, 1996; White, 1992; Krishna and White, 1996). Despite the fact that quinine is one of the oldest drugs (at least 350 years) in the pharmacopoeia and the most widely used antimalarial drug, the detailed metabolism of quinine and cytochrome P450(CYP) isoform(s) involved have been only recently elucidated: the formation of 3-hydroxyquinine from quinine is the major metabolic pathway (Wanwimolruk et al., 1995; Wanwimolruk et al., 1996). It is catalyzed mainly by CYP3A4 (Zhao et al., 1996; Zhang et al., 1997) and to a minor extent by CYP2C19 (Zhao et al., 1996) in human liver microsomes. Our recent study has shown that CYP3A/CYP3A also plays a dominant role in the formation of 3-hydroxyquinine from quinine in mouse, rat, and dog liver microsomes, and 3-hydroxyquinine is the main metabolite of quinine in these animal livers (Zhao and Ishizaki, in press).

On the other hand, etoposide, a commonly used anticancer agent with a broad range of antitumor activity, is claimed to be metabolized largely via CYP3A4 by 3'-demethylation in human liver microsomes and its metabolite has some antitumor activity (Relling et al., 1992). Like quinine, etoposide also possesses a relatively low therapeutic index with some adverse reactions in cancer patients (e.g., leukopenia, platelet deficiency, thrombocytopenia, and leukopenia) (Kobayashi and Ratain, 1994). Therefore, from a theoretical point of view, a drug-drug interaction might occur when quinine and etoposide are co-administered in patients with cancer who are living in malaria endemic areas such as Southeast Asia, South America, and East Africa (i.e. 300 to 500 million new cases of malaria every year). More importantly, if a mutual inhibition between quinine and etoposide in human liver microsomes would occur, it should provide further evidence for the role of CYP3A involvement in the metabolism of both quinine and etoposide. Based on the background as discussed above, we conducted this study to assess the mutual interaction potential of quinine and etoposide in vitro, as well as to confirm further that etoposide and quinine are metabolized mainly via CYP3A in human liver microsomes.

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'-demethyltoposide and the internal standard (4'-demethyllepodaiphylotoxin-9-(4,6,0-propyliodone-β-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).

This study was supported by a grant-in-aid from the Ministry of Health and Welfare, 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'-demethyletoposide 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 (Taitec, 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'-demethyletoposide 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, 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 Vₘₐₓ) were estimated according to the Eadie-Hofstee equation by use of the nonlinear least-squares regression analysis program, MULTI (Yamaoka et al., 1981).

Results and Discussion

Chromatograms and Assessment of Incubation Conditions. With the described chromatographic conditions, no interfering peaks for 3-hydroxyquinine, 3'-demethyletoposide, or the internal standard were present in the incubation mixture. The formation of both 3-hydroxyquinine from quinine and 3'-demethyletoposide from etoposide were time-, NADPH- and microsome-dependent, suggesting the possible involvement of P450(s) in their 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'-demethyletoposide 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 µM (fig. 1). The mean maximum inhibition produced by etoposide (100 µM) 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. 2. 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 µM. The mean maximum inhibition produced by quinine (100 µM) 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 µM (2.5–20 µg/ml) (White, 1988; White, 1992) and up to 80 µM (50 µg/ml) (Hande et al., 1984), respectively, which are near or within the respective IC$_{50}$ value (90 µM by quinine and 65 µM by etoposide), 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′-demethyltoposide 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 µM) (Newton et al., 1995; Maurice et al., 1995). Thus, the concentrations of ketoconazole used in this study were < 2 µM (i.e., 0.1 and 1.0 µM).

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 chemical inhibition experiments presented here (a maximal inhibition of 90%) and chemical inhibition experiments performed previously (a maximal inhibition of 60%). 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 inhibition versus 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 (Zhao 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.
MUTUAL INHIBITION OF QUININE-ETOPOSIDE METABOLISM IN VITRO

Fig. 4. Effects of TAO and ketoconazole on quinine 3-hydroxylation (■) and etoposide 3’-demethylation (□) in human liver microsomes.

Data are the mean ± SD values of experiments performed with microsomes obtained from three to four different livers.

Acknowledgments. We thank Dr. P. Winstanley, University of Liverpool, UK, for the generous donation of 3-hydroxyquinine. We also acknowledge Dr. Wanwimolruk, University of Otago, New Zealand, for supplying the column used as an analyzing tool of 3-hydroxyquinine and Hunan Medical University, China, for supporting Dr. Zhao’s training in Japan. This study was supported by a grant-in-aid from the Ministry of Health and Welfare and by a postdoctoral fellowship training program from the Bureau of International Cooperation, International Medical Center of Japan, Tokyo, Japan.

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