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Rat liver microsomal and mitochondrial metabolism of primaquine *in vitro*[1]

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ABSTRACT The profiles of major metabolites of primaquine (PQ) produced from liver microsomal (MC) and mitochondrial (MT) metabolism were investigated *in vitro* by silica gel thin layer and reverse phase high perssure liquid chromatography (HPLC). The results indicated that 5-hydroxy primaquine (5-OH PQ) and carboxyprimaquine (CPQ) were simultaneously produced by either microsomes or pure mitochondria preparations. However, the quantitative study showed that microsomes produced approximately 19 times more 5-OH PQ but only 1/34 of the CPQ by mitochondria.

KEY WORDS primaquine; liver microsomes; liver mitochondria; thin layer chromatography; high pressure liquid chromatography; drug metabolic detoxication

Primaquine (PQ) has remained the antimalarial of the first choice for radical treatment of vivax malaria and all tissue phase of *Plasmodium parasites* despite its manifold toxic effects, among which hemolysis in G-6-PDH deficient subjects is the most serious one. The toxic effects of PQ are attributable to its various metabolites and/or reactive intermediates[1,2].

To date, 2 metabolic pathways of PQ have been identified. One pathway leads to formation of some hydroxylated derivatives of PQ at 5- and/or 6-position of quinoline nucleus, which are more potent methemoglobinogenic[1,2]. But there has been little evidence to prove their existence in human. The another pathway produces carboxyprimaquine (CPQ), which was detected as the major metabolite of PQ in human plasma with neither antimalarial nor methemoglobinogenic activities[3,4]. This study was designed to investigate the profiles of metabolites produced by liver microsomes and mitochondria.

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MATERIALS AND METHODS

Shimadzu LC-6A high pressure liquid chromatograph (HPLC), sample injector 7125, Shimadzu HPLC monitor model SPD-6AV, and Shimadzu recorder & data processor model C-R6A.

Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), and glucose-6-phosphate dehydrogenase (G-6-PDH) were purchased from Sigma. PQ (Southwest Second Pharmaceutical Factory) was purified (99% pure) from re-crystallization by Department of Pharmaceutical Chemistry, Institute of Parasitic Diseases. The standard compound of 5-OH PQ and other 5 putative metabolites of PQ were kindly granted by Dr DE Davidson Jr, the Steering Committee on Malaria Chemotherapy, WHO. CPQ was a gift from Dr J K Baker, School of Pharmacy, University of Mississippi, USA.

Liver microsomal and mitochondrial metabolism *in vitro* The preparation of rat liver microsomes and the operation of *in vitro* metabolism systems followed the routine of our laboratory[5,6]; the preparation of pure mitochondria from rat liver homogenate followed our method previously reported[7]. All samples were wrapped with aluminum foil to shelter from light during the whole experiment.

Thin layer chromatography The incubation mixtures after microsomal or mitochondrial metabolism *in*

vitro were extracted with *n*–BuOH, concentrated under N_2 gas, and chromatographed on silica gel GF 254 pre–coated thin layer plates (produced by Fu Sang Biochemical Reagents Factory and supervised by the Shanghai Institute of Materia Medica, Chinese Academy of Sciences). The model compounds of PQ, 5–OH PQ, and CPQ were simultaneously co–chromatographed for comparison. The composition of the solvent system was benzene : MeOH : glacial HAc, 66 : 33 : 1.

Reverse phase HPLC analysis In view of the variety of PQ metabolites, 2 pre–treatment systems were used. System I: The incubation mixture after metabolism *in vitro* was extracted with cold (4℃) *n*–BuOH. The precipitated proteins were removed by centrifugation at 3000 × *g* for 30 min. The supernatant in organic phase was concentrated under N_2 gas. The residue was washed with Tris buffer 0.2 $mol \cdot L^{-1}$ at pH 7.4, then re–extracted with CH_2Cl_2. The residue of re–concentrated organic phase was dissolved in 1 ml MeOH for HPLC analysis. System II: The incubation mixture was extracted with cold acetyl acetate and citric acid buffer (1:1) at pH 3.2. The precipitated proteins were removed as in System I. The supernatant in organic phase was concentrated under N_2 gas. The residue was washed with citric acid buffer at pH 3.2, then re–extracted with acetyl acetate. The organic phase was re–concentrated and the residue was dissolved in 1 ml MeOH for HPLC analysis.

The column was Shim–Pack, CLC–ODS, 15 × 0.6 cm. Mobile phase System I: 1.6 ml *n*–octyl amine, 2 ml glacial HAc in 1 L of 48% $MeOH-H_2O$ solution (pH 4.5); System II: 5.0 g $K_2HPO_4 \cdot 3H_2O$ and 5.8 g KH_2PO_4 in 1 L of redistilled water, 350 ml of phophate buffer and 650 ml of MeOH mixed, and 1.6 ml of *n*–octyl amine added (pH 9.0). Flow rate: $1\ ml \cdot min^{-1}$, uv detecting at 265 nm.

Quantitative comparison The standard solutions of PQ 20 $ng \cdot \mu l^{-1}$, 5–OH PQ 12 $ng \cdot \mu l^{-1}$, and CPQ 12 $ng \cdot \mu l^{-1}$ were prepared and analyzed by the 2 HPLC systems. The interrelationship between peak area and absolute content was determined according to the absolute standard curve with *P* value corrected by a data processor model C–R6A. Each incubation mixture after MC or MT metabolism was divided into 2 equal aliquots, then pretreated and HPLC analyzed with System I or II. The content of each metabolite was calculated according to the corresponding peak area, and the $ng \cdot ml^{-1}$ values of metabolites were determined by the sample volume (5 μl) of each injection. The quantitative comparison of metabolites was accomplished by calculating the production rate from MC or MT metabolism, both starting from 1 mg of PQ.

RESULTS

Metabolite profile of PQ on thin layer chromatogram The incubation mixture of MC metabolism developed into 4 spots and / or zones (Fig 1). Spots A, B, and C corresponded to CPQ, 5–OH PQ and unmetabolized PQ, respectively; zone D may be some other unidentified metabolites. The incubation mixture of MT metabolism developed into only 3 spots and / or zones. The spots A, C, and zone D were same as that of

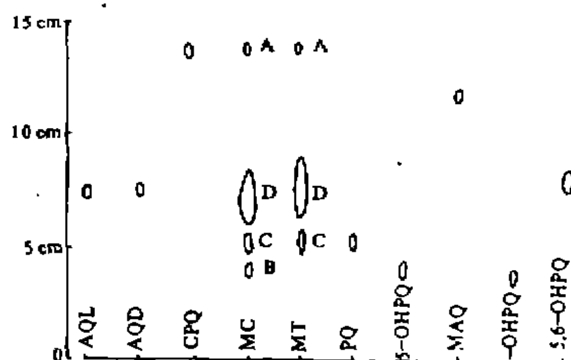

Fig 1. Thin layer chromatogram of primaquine (PQ) and its 7 putative model metabolites and extracts from liver microsomal (MC) and mitochondrial (MT) metabolism mixtures. A: carboxyprimaquine (CPQ); B: 5–hydroxy primaquine (5–OHPQ); C: primaquine; D: unidentified metabolites of primaquine; AQL: 6–hydroxy–8–aminoquinoline; AQD: 5,6–dihydroxy–8–aminoquinoline; 5–OHPQ: 5–hydroxyprimaquine; MAQ: 6–methoxy–8–aminoquinoline; 6–OHPQ: 6–hydroxyprimaquine; 5,6–OHPQ: 5,6–dihydroxyprimaquine.

MC, but no spot B was seen.

Metabolite profile of PQ on reverse phase HPLC A good seperation of major metabolites of PQ was achieved by the pre-treatment System I and II with the corresponding mobile phases I and II on HPLC. The quinoline-ring oxidative metabolites, such as 5-OH PQ, were mainly isolated by System I. The peak times were 3 min for 5-OH PQ and 5 min for PQ. While the side-chain oxidative deamination metabolite-CPQ was isolated by System II. The peak times were 4.6 for CPQ and 7.1 min for PQ (Fig 2).

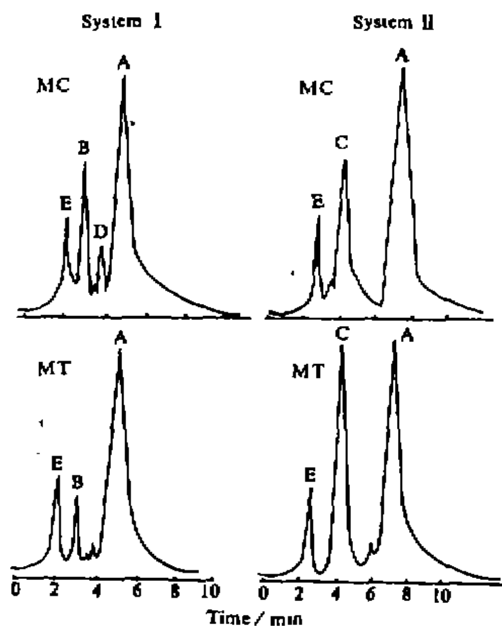

Fig 2. Reverse-phase HPLC chromatogram of PQ metabolites. Peak A: PQ remained. B: 5-OHPQ. C: CPQ. D: Unidentified. E: Solvent.

Quantitative comparison Both 5-OH PQ and CPQ formed from *in vitro* metabolism of PQ (Tab 1). From MC, 5-OH PQ was produced at a production rate of 1.522% of 1 mg starting material, while CPQ was produced only 0.077% of 1 mg starting material. From MT, CPQ was produced at a production rate of 2.625%, while 5-OH PQ was produced only 0.08% of 1 mg starting material. The differences of production rates of 5-OH PQ and CPQ between MC and MT were significant ($P<0.01$).

Tab 1. Production rates of 5-hydroxyprimaquine (5-OHPQ) and carboxyprimaquine (CPQ) from MC or MT metabolism. *n*: **repeated times of** *in vitro* **metabolism;** $\bar{x} \pm s$, $^{***}P<0.01$, **5-OHPQ** *vs* **CPQ from either MC or MT.**

	n	5-OHPQ	*n*	CPQ
ng from HPLC				
MC	6	76±28	4	3.8±2.1
MT	3	4.0±2.9	3	131±22
Production rate / %				
MC	6	1.52±0.56***	4	0.08±0.04***
MT	3	0.08±0.06***	3	2.63±0.04***

DISCUSSION

In present study, we found that two types of major metabolites of PQ were produced at different subcellular compartments, i e, 5-OH PQ at MC while CPQ at MT.

Theoretically, a model scheme of 2 major metabolic pathways of PQ is proposed in Fig 3 that includes 3 enzyme-mediated routes. 1 quinoline-ring oxidation pathway which produces 5-OH PQ; 2 side-chain N-oxidation pathway which finally yields CPQ; : side-chain oxidative deamination which also forms CPQ. It is assumed that route I is catalyzed by cytochrome P-450 which is the major oxidative enzyme complexes in microsomes and route 3 is catalyzed by monoamine oxidase which is richly contained in mitochondria, while both MC and MT might be involved in route 2.

MT is one of the major subcellular targets for PQ[8-10]. Therefore, though CPQ was

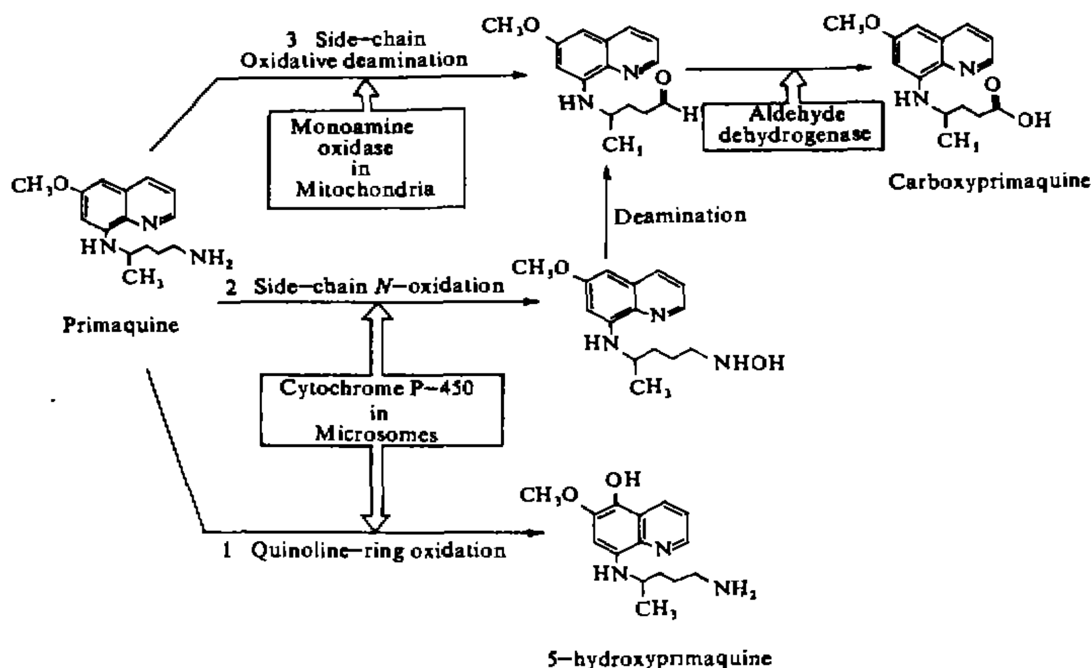

Fig 3. Suggested metabolic pathways of primaquine.

produced at MT as a detoxication metabolite, there might be some biological reactive intermediates formed in the process of MT metabolism. Since most of those intermediates are much unstable with higher chemical reactivity, more sensitive analyzing methodology should be developed to undertake the detailed studies.

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伯喹的大鼠肝微粒体和线粒体体外代谢

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提要 以硅胶薄层色谱法及反相高压液相色谱法研究了伯喹在肝微粒体和线粒体体外代谢的主要代谢产物谱. 结果提示，微粒体或线粒体制品均可同时产生5-羟基伯喹和羧基伯喹；但定量研究表明微粒体产生的5-羟基伯喹量比线粒体的大19倍，而前者产生的羧基伯喹仅为后者的1/34.

关键词 伯喹；肝微粒体；肝线粒体；薄层色谱法；高压液相色谱法；药物代谢解毒

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Antitumor effects of new-type recombinant interleukin-2[1]

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ABSTRACT Two new human recombinant interleukin-2 (rIL-2), 125-Ser-rIL-2, and 125-Ala-rIL-2, were generated by protein engineering technique. Both of them maintained the proliferation of natural killer (NK) cells, CTLL-2 cells and their long-term propagations. The mutated new rIL-2 also enhanced the bioactivity of NK cells and the cytotoxicity of tumor-infiltrating lymphocytes (TIL) against the target tumor cells. The above results were all compared with that of the native rIL-2 and a similarity between them was found, which indicates that new type rIL-2 could be used for adoptive immunotherapy of malignant diseases.

KEY WORDS interleukin-2; protein engineering; natural killer cells; tumor-infiltrating lymphocytes; cultured tumor cells

Interleukin-2 (IL-2) is an immunoregulatory lymphokine secreted by antigen-stimulated T cells which can enhance NK activity, stimulate its differentiation and proliferation, induce lymphokine-activated killer (LAK) cells and tumor-infiltrating lymphocytes[1-3]. Since native IL-2 from cell culture is difficult to be obtained and purified, the study for clinical application of IL-2 has been quite limited. Fortunately the development of recombinant IL-2 (rIL-2) has greatly changed this situation[4]. Native rIL-2 contains 3 Cys. Among them 58-Cys and 105-Cys form a disulfide bond which is necessary for its activity[5, 6]. The 125-Cys is free and is liable to mismatch with other Cys. To prevent this mismatch 125-Cys was substituted with Ser and Ala by means of protein engineering in Shanghai Institute of Biochemistry, Chinese Academy of Sciences (CAS) and two new type of rIL-2 with 125-Ser and 125- Ala was elaborated. In this report the biological features, especially the antitumor effects, of the 2 new rIL-2 were studied and compared with that of the native one.

MATERIALS AND METHODS

C3H mice, weighing 15.0 ± *s* 0.8 g, were provided by the Experimental Animal Center of Shanghai

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