CYTOCHROMES P450 INVOLVED IN CYCLOPHOSPHAMIDE, PACLITAXEL AND DOCETAXEL METABOLISM IN RATS

Lucie BOŘEK-DOHALSKÁ[b1], Ivan GUT[a1,*], Pavel SOUČEK[a2], Zdeněk ROTH[a3] and Petr HODEK[b2]

[a] *National Institute of Public Health, Center of Industrial Hygiene and Occupational Diseases, Biotransformation Group, 100 42 Prague 10, Šrobárova 48, Czech Republic; e-mail: [1] ivangut@szu.cz, [2] psoucek@szu.cz, [3] roth@szu.cz*

[b] *Department of Biochemistry, Faculty of Science, Charles University, 128 40 Prague 2, Hlavova 2030, Czech Republic; e-mail: [1] dohalska@yahoo.com, [2] hodek@prfdec.natur.cuni.cz*

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We investigated involvement of cytochromes P450 (CYPs) of rat liver microsomes in metabolism of two anticancer drugs, paclitaxel (PCT) and docetaxel (DTX), by an indirect method. This method is based on the presumption that the compound competitively inhibiting oxidation of the CYP-selective substrate should also be a substrate for the CYP enzyme. The validity of this approach was confirmed using the model drug, cyclophosphamide (CPA). Indeed, CPA competitively inhibited oxidation of substrates specific for CYP2B1 and CYP3A1/2, enzymes previously reported to be capable of metabolizing CPA. Using this method, we identified CYP enzymes participating in PCT and DTX metabolism. The CYP2D1/2/3 and CYP3A1/2 are enzymes oxidizing PCT while CYP3A1/2 and CYP2E1 are responsible for metabolism of DTX. Here, we report a suitable method serving for easy and fast estimation of CYP enzymes involved in drug metabolism.

Key words: Cytochrome P450; Cyclophosphamide; Paclitaxel; Docetaxel; Taxanes; Enzyme inhibition; Metabolism; Antitumor drugs; Biotransformations.

Clinical effectiveness of anticancer drugs depends on the delicate balance between their cytotoxicity for tumor and/or normal cells and their metabolic activation or inactivation. The exact elucidation of biotransformation of these drugs and in particular enzymes catalyzing their metabolism is, therefore, crucial for modulation of their efficiencies.

Cytochromes P450 (CYP) (EC 1.14.14.1.)[+] are principal enzymes metabolizing important anticancer drugs such as cyclophosphamide (CPA),

+ *Abbreviations*: CYP, cytochrome P450 (EC 1.14.14.1); CPA, 2-[bis(2-chloroethyl)amino]-2*H*-1,3,2-oxazafosfinan 2-oxide (cyclophosphamide); DTX, docetaxel; PCT, paclitaxel; CLZ, 5-chloro-2*H*-1,3-benzoxazol-2-one (chlorzoxazone); 7-ER, 7-ethoxy-3*H*-phenoxazin-3-one (7-ethoxyresorufin); 7-PR, 7-pentyloxy-3*H*-phenoxazin-3-one (7-pentoxyresorufin).

ifosfamide, hexamethylmelamine, etoposide, thiotepa, tamoxifen, 17-ethinylestradiol, and the recently introduced paclitaxel (PCT, Taxol®) and docetaxel (DTX, Taxotere®) (Fig. 1). Thus, activities of these enzymes can significantly modify both their pharmacological efficiency and toxicity.

CPA is a drug often used for treatment of various human tumors. The alkylation of DNA is the main mechanism of the CPA antitumor action. To exert anticancer activity, CPA has to be activated by CYP enzymes to 4-hydroxy derivative[2–4]. This reaction is catalyzed by rat CYP2B1, 2C6 and 2C11 (ref.[5]) and human CYP2A6, 2B6, 2C8 and 2C9 (ref.[6]). On the other hand, CPA is *N*-dechloroethylated to a neurotoxic metabolite chloroacetaldehyde having no antitumor activity[2,7]. This reaction is catalyzed by rat and human CYP3A (refs[5,9]).

PCT and DTX provide effective therapy of breast, ovarian and lung cancer[1]. These taxoids exert a unique mechanism of action – inhibition of microtubule disassembly and thereby cell division and proliferation followed by inducing of apoptosis[1,10,11].

6α-Hydroxy derivative of PCT is the main metabolite of this anticancer drug in humans. CYP2C8 is responsible for production of this metabolite[12,13]. 3′-(4-Hydroxyphenyl) derivative of PCT was found as a minor metabolite. Formation of this product is catalyzed by CYP3A (refs[14,15]). Different metabolic pathways were determined for this drug in rats. The major PCT metabolite in humans, 6α-hydroxypaclitaxel, is not formed by any CYP in rats. In this species, PCT is preferentially metabolized to 2′- and 3′-(4-hydroxyphenyl) derivative of PCT by CYP3A (ref.[16]). Other PCT products are generated, both in humans and rats, and their structures and CYPs involved in their formations have not been determined as yet[17].

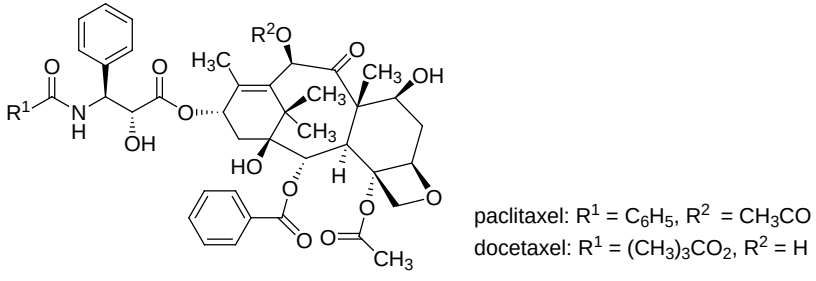

FIG. 1
Structures of taxanes. Paclitaxel: $R^1 = C_6H_5$, $R^2 = CH_3CO$; docetaxel: $R^1 = (CH_3)_3CO_2$, $R^2 = H$

In contrast to PCT, DTX produced a very similar metabolic pattern in humans, rats, mice, rabbits and dogs[18,19]. The major metabolic pathway is the hydroxylation of *tert*-butyl group at the C-13 side chain of the taxane skeleton, yielding 9′-hydroxy derivative of DTX. This metabolite is thereafter oxidized to form oxazolidinedione and two isomers of hydroxyoxazolidine. Preliminary reports[19–23] showed that formation of the above metabolic products seems to be catalyzed by CYP3A. It is evident that metabolism of PCT and DTX as well as CYPs involved in their oxidation are not understood well. Therefore, the present study is aimed at extending our knowledge on CYPs responsible for metabolism of both these effective anticancer drugs.

EXPERIMENTAL

Chemicals

Chemicals of highest purity: $MgCl_2$, Tris, KCl, NADPH, $NADP^+$, glucose 6-phosphate, resorufin (7-hydroxy-3*H*-phenoxazin-3-one), its ethyl derivative (7-ethoxy-3*H*-phenoxazin-3-one) and pentyl derivative (7-pentyloxy-3*H*-phenoxazin-3-one), chlorzoxazone (5-chloro-2*H*-1,3-benzoxazol-2-one), β-naphthoflavone (3-phenyl-1*H*-benzo[*f*]chromen-1-one), 6β-hydroxytestosterone (6β-hydroxy-4-androsten-17β-ol-3-one), pregnenolone 16α-carbonitrile (5-pregnen-3β-ol-20-one 16α-carbonitrile) and cyclophosphamide were obtained from Sigma Chemical Co. (St. Louis (MO), U.S.A.). Glucose 6-phosphate dehydrogenase was obtained from Boehringer (Mannheim, Germany). Bufuralol (α-{[(1,1-dimethylethyl)amino]-methyl}-7-ethyl-2-benzofuranmethanol) and its 1′-hydroxy derivative were obtained from Gentest Corp. (Woburn (MA), U.S.A.). Testosterone, dichloromethane and methanol were products of Merck (Darmstadt, Germany). 6-Hydroxy derivative of CLZ was synthesized[24] in the Research Institute of Pharmacy and Biochemistry, Prague, Czech Republic. Paclitaxel was obtained from Fluka Chemie AG (Switzerland) and docetaxel was a kind gift from Rhone–Poulenc Rorer (France).

Animals, Pretreatments and Preparation of Microsomes

Adult male Wistar rats (200–260 g, VELAZ, Czech Republic) were fed *ad libitum* on pellet chow and water one week before use. Four groups of rats (N = 7–8) were specifically pretreated to induce high levels of CYP1A1/2, 2B1/2, 2E1 and 3A1/2 in rat liver as reported before[25]. Control microsomes were prepared from untreated rats. The animals were killed and livers in particular groups were pooled for preparation of microsomes by centrifugation as described before[25]. Microsomes were stored in aliquots at –80 °C until use. Protein concentration was determined according to Lowry and co-workers[26] using serum albumin as a standard. The total CYP content was estimated by the method of Omura and Sato[27].

Enzyme Assays

CYP activities were studied with microsomes from rats pretreated with CYPs abovementioned inducers of. CYP2E1 activity[28,29] was assayed as CLZ 6-hydroxylation according

to Peter and collaborators[30]. The reaction mixture composed of 10–100 μM CLZ as a substrate, the NADPH-generating system (10 mM $MgCl_2$, 10 mM glucose 6-phosphate, 1 mM $NADP^+$, 0.5 U per ml of glucose 6-phosphate dehydrogenase) and microsomes containing 100 pmol of CYP was incubated for 15 min. 7-ER *O*-deethylase (CYP1A1/2 activity) with 1.75 μM substrate and 7-PR *O*-deethylase (CYP2B1/2 activity) with 0.25-5 μM substrate were assayed with 0.25 mM NADPH and microsomes containing 100 pmol of CYP for 20 min (ref.[31]). Testosterone oxidation[32] (CYP3A activity) was determined with 10–100 μM substrate, the NADPH-generating system (see above) and microsomes containing 100 pmol of CYP for 15 min and bufuralol 1′-hydroxylation[33,34] (rat CYP2D1/2/3) with 2.5–25 μM substrate, the NADPH-generating system and microsomes containing 100 pmol of CYP for 20 min. All incubations were carried out in a total volume of 0.5 ml, at 37 °C in a shaking water bath and in the presence or absence of CPA (200–1 500 μM), PCT (25–200 μM) or DTX (25–100 μM) to determine the inhibition effect. Microsomes were preincubated with or without the anticancer drugs (10 min at 37 °C with the NADPH-generating system) to evaluate the reversibility and/or irreversibility of inhibition.

Data and Statistical Analysis

For all assays, metabolite levels were determined using a standard curve generated from the authentic standard. Data are means ±SEM. Effect of inhibitor concentration was analyzed by regression analysis, K_i values were calculated according to Dixon[36].

RESULTS AND DISCUSSION

To estimate the metabolism of various drugs directly is often very difficult, because their metabolic pathways and formed products are not identified. Therefore, several indirect methods suitable for determination of CYPs involvement in drug metabolism are frequently utilized instead of direct methods[35]. Here, we present and verify one of such methods. It is based on the following assumptions: if oxidation of a selective substrate of a particular CYP isozyme is competitively inhibited by the tested compound (drug), the CYP should also be able to oxidize such a compound. To confirm the validity of the method, CPA, the metabolism of which as well as the CYPs oxidizing this drug are well known, was utilized as a model compound. Rat hepatic microsomes containing the CYP-dependent enzyme system were used in the study.

Experiments were carried out in two parallel protocols. In the first one, the reaction mixture containing all components (microsomes, the NADPH-generating system and the CYP selective substrate) was incubated immediately after the addition of CPA. In the other protocol, CPA was incubated only with microsomes and the NADPH-generating system (10 min) before the addition of a CYP-specific substrate. The knowledge about effects of the above mentioned preincubation on inhibition efficiencies is necessary for (i) determination of reversibility or irreversibility of inhibition and (ii) for

investigation whether metabolic products of the drug may bind better to the CYP enzyme active center than the parent drug. The detailed kinetic studies were performed and the efficiency of inhibition was characterized by K_i values (Table I).

CPA competitively inhibited oxidation of CYP2B1- and CYP3A1/2-selective substrates, while the activities of CYP1A1/2, CYP2D1/2/3 and CYP2E1 were not affected (Table I). This is in accordance with results reported by Waxman and coworkers[5,6,8]. Therefore, the method used in the present paper seems to be suitable for purposes we require (identification of CYPs oxidizing anticancer drugs).

CPA preincubated in the reaction mixture led to a stronger inhibition of specific activities of CYP2B1 and CYP3A1/2. Likewise, the K_i values (see Table I) decreased significantly after CPA preincubation. Also in this case, the CYP inhibition by CPA was competitive. This finding indicates that CPA acts as a reversible CYP inhibitor and its products formed in CYP2B1- or CYP3A1/2-mediated metabolism are able to bind to the enzyme active center better than the parent drug.

The described method was further used for determination of CYPs participating in metabolism of other anticancer drugs, paclitaxel and docetaxel. As mentioned above, their metabolism and CYPs oxidizing them have not been understood in detail as yet.

PCT affected CYP2D1/2/3 and CYP3A1/2 specific activities, while CYP1A1/2, CYP2B1 and CYP2E1 were not changed. The pattern of inhibi-

TABLE I
The effect of cyclophosphamide, paclitaxel and docetaxel on CYP-specific activities

Anticancer drug	Inhibition constant K_i, μM					
	CYP1A1/2	CYP2B1	CYP2D1/2/3	CYP3E1	CYP3A1/2	
CPA	–[a]	77.8 ± 23.2[b]	–[a]	–[a]	4 640 ± 700[b]	
	–[a]	265.5 ± 37.5[c]	–[a]	–[a]	2 060 ± 350[c]	
PCT	–[a]	–[a]	189.9 ± 38.0[b]	–[a]	55.5 ± 8.6[b]	147.8 ± 19.4[b]
	–[a]	–[a]	212.3 ± 16.7[c]	–[a]	56.7 ± 4.9[c]	106.4 ± 10.6[c]
DTX	–[a]	–[a]	–[a]	306.4 ± 8.3[b]	17.2 ± 2.6[b]	
	–[a]	–[a]	–[a]	149.6 ± 10.8[c]	17.6 ± 1.8[c]	

[a] No inhibition. [b] No preincubation. [c] 10 min preincubation.

tion determined from Dixon plots indicates that interaction of CYP2D1/2/3 and CYP3A1/2 with PCT is consistent with a competitive type of inhibition without any changes in K_i values after preincubation (Table I). Dixon plots of CYP3A1/2 inhibition by PCT were not linear (Fig. 2). Thus, two K_i values were found, one of them being similar to K_i obtained for inhibition of CYP2D1/2/3. These results confirm that PCT is converted by CYP3A1/2 (as reported previously[16]). Moreover, they strongly suggest a role of other CYPs, namely CYP2D1/2/3, in PCT metabolism.

DTX competitively inhibited specific activities of CYP2E1 and CYP3A1/2, while activities of CYP1A1/2, CYP2B1 and CYP2D1/2/3 were not affected. The preincubation of DTX did not influence the degree of CYP inhibition (Table I). The obtained data confirm the role of CYP3A1/2 in the DTX metabolism. Moreover, we identified an additional CYP enzyme, which seems to be implicated in its oxidation (CYP2E1). This new finding, however, should be confirmed in future. Detailed study of DTX metabolism is under way in our laboratory.

The present paper clearly shows that the evaluated indirect method is entirely suitable for identification of CYPs metabolizing drugs. Using this method, we confirmed that the CYPs reported earlier (CYP2B1 and 3A1/2) are involved in the metabolism of CPA (refs[5–7]). Moreover, we extended the knowledge on CYPs implicated in metabolism of other two anticancer drugs, PCT and DTX, the information on which was rather limited. There-

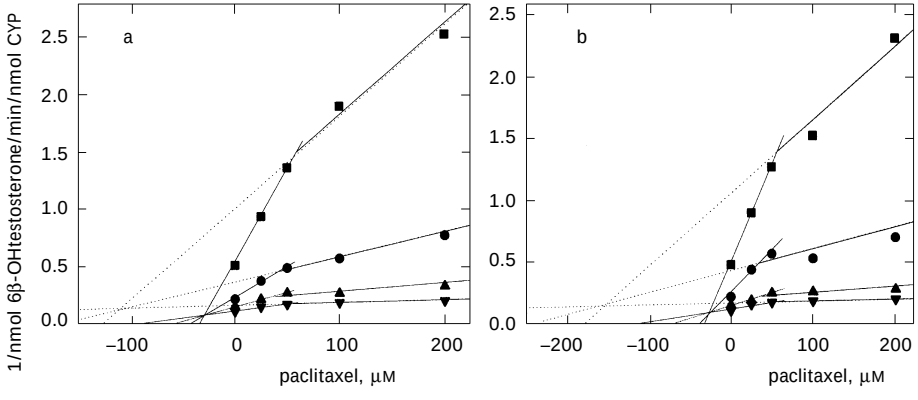

FIG. 2

Dixon plots of the effect of paclitaxel on CYP3A activity (testosterone 6β-hydroxylation) in rat liver microsomes. Testosterone concentrations were 10 (❐), 20 (●), 40 (Δ) and 80 μM (∇). Rat microsomes were preincubated with (a) or without (b) paclitaxel

fore, we propose this method as an easy and fast screening for identification of CYPs responsible for drug metabolism.

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