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METHODS

The Use of Human Liver Cell Model and Cytochrome P450 Substrate—Inhibitor Panel for Studies of Dasatinib and Warfarin Interactions

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The possibility of interactions between warfarin and dasatinib and their interactions with other drugs metabolized by cytochrome P450 isoform CYP3A4 was demonstrated using a previously created cytochrome P450 substrate—inhibitor panel for preclinical *in vitro* studies of drug biotransformation on a 3D histotypical microfluidic cell model of human liver (liver-on-a-chip technology). Dasatinib and warfarin are inhibitors of CYP2C19 isoform and hence, can interfere the drugs metabolized by this isoform. Our findings are in line with the data obtained on primary culture of human hepatocytes and suggest that the model can be used in preclinical *in vitro* studies of drugs.

Key Words: *biotransformation; microbioreactor; cell model of the liver; drug interactions*

Cytochrome P450 (CYP450) isoforms are the main enzymes of phase 1 xenobiotic biotransformation, including drugs. Studies of biotransformation routes and interactions between the drugs metabolized in the liver are carried out on subcellular fractions (microsomes) and primary cultures of human and animal hepatocytes. However, comparison of the expression profiles and activities of nine CYP450 isoforms in liver microsomes of mice, rats, rabbits, dogs, monkeys, and humans showed that none of the species could describe even tentatively the profile of these enzymes activities in humans [4]. Differences in the expression and activities of CYP450 isoforms in various species lead to the formation of different metabolites in animals and humans and to changes in the rate of drug biotransformation [5].

Isoforms CYP3A4 and CYP2C9 metabolize more than 50% known drugs [12]. Isoform CYP3A11 is the closest homologue of the mouse CYP3A4. Comparison of these human and mouse enzymes has detected differences in their specificity and activity [4]. It should be noted that some drugs (for example, rifampicin) induce CYP3A4 in humans and cause no changes in CYP3A11 expression in mice. The structural analog of human CYP2C9 isoform in mice is CYP2C29, but differences in substrate specificity of these isoforms do not allow extrapolation of the data obtained on animal models [4]. This fact necessitates the development of new methods for toxicity evaluation by highly informative *in vitro* tests based on the use of modern cell technologies [3,9]. The most promising tests for prediction of hepatotoxicity of currently used or newly created drugs are alternative *in vitro* tests with the use of maximally adequate microfluidic cell model of human liver (liver-on-a-chip technology) [2,10,13].

A specific substrate—inhibitor panel for four most important CYP450 isoforms has been created on the bases of a microbioreactor for studies of drug compound biotransformation pathways, specifically, for

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TABLE 1. Substrate—Inhibitor Panel for the Development of Methodology for Preclinical Studies of Drug Biotransformation *In Vitro*

CYP450 isoform	CYP2B6	CYP2C9	CYP2C19	CYP3A4
Relative expression, %	2-10	20		40
Relative content of metabolized substrates, %	3-12	12		50
Substrate	Bupropion	Tolbutamide	Omeprazole	Testosterone
Substrate concentration, μM	10	40	20	10
Inhibitor	PPP (2-phenyl-2-(1-piperidine)-propane	SF (Sulfafenazole)	NBN ((+)-N-3-benzyl-nirvanol)	KZ (Ketoconazole)
Inhibitor concentration, μM	30	5	1	1

detection of the CYP450 isoform involved in the drug metabolism (Table 1). The four CYP450 isoenzymes selected for creation of the panel constitute ~70% expressed forms of CYP450 and metabolize about 70% of all drugs.

Our aim was analysis of the interactions between dasatinib and warfarin using the CYP450 substrate—inhibitor panel, to be more precise, and detection of the CYP450 isoenzymes metabolizing these compounds and the interactions between the test compounds and panel substrates.

MATERIALS AND METHODS

Cells HepaRG were cultured in complete nutrient medium (CNM): William's Medium E (Gibco), 2 mM L-glutamine (Gibco), 10% fetal calf serum (FCS; HyClone), 5 μg/ml human recombinant insulin (Gibco), 5×10^{-5} M hydrocortisone hemisuccinate (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). The cells differentiated in CNM with 2% DMSO for 14 days, after which spheroids were formed for 5 days in 96-well low-adhesion round-bottom plates. The spheroids were then transferred into serum-free nutrient medium and further incubated for 24 h. The composition of serum-free medium was as follows: William's E with L-glutamine (Gibco), insulin—transferring—selenium (1×; ITS; Thermo Scientific), 1 μg/ml BSA (Sigma), 5×10^{-5} M hydrocortisone hemisuccinate (Sigma), non-essential amino acids (1×; NEAA; Thermo Scientific), and 1% penicillin/streptomycin (Gibco). The spheroids were cultured in replaceable cell blocks of the microbioreactor [1].

For testing the expression of CYP450 isoforms, the cells were lyzed in Qiazol (Qiagen) after culturing, RNA was isolated, and gene expression was analyzed by real-time PCR (Table 2) as described previously [11].

Spheroids (n=100) were put in wells of replaceable cell blocks (RCB), nutrient medium was then replaced with 125 μl medium containing CYP450 substrate mixture (10 μM bupropion, 10 μM testosterone, 40 μM tolbutamide, 20 μM omeprazole) without and with each of the specific inhibitors in the concentrations shown in Table 1, after which the test drug (warfarin or dasatinib) was added (1 μM). The test substance (warfarin or dasatinib, 1 μM) without inhibitor/substrate served as the control. Culturing was carried out for 24 h at 5 Hz valve cycling frequency and ±10 kPa pressure. Then, the medium (100 μl) was sampled from each RCB and mixed with 35 μl cold (-20°C) acetonitrile. Chromato-mass-spectrometric analysis was then carried out for measuring 7-hydroxywarfarin and 4'-hydroxydasatinib levels. The

TABLE 2. Primers Used for Evaluation of Gene Expression

Gene	Forward primer	Reverse primer
Cyp3A4	3'-GATTCCAAGCTATGCTCTTCAC-5'	3'-TTCTTGCTGAATCTTTCAGGG-5'
Cyp2C9	3'-TTGACTTGTTTGGAGCTGG-5'	3'-TGGACTTTAGCTGTGACCT-5'
RPL23A	3'-ctggaagaggctgtgtatgaa-5'	3'-tagtagatgggtgtgtgaggac-5'
RAB10	3'-agtggatggcaactgatggaac-5'	3'-agacccctgtaaaagagcccaac-5'
UBE2Q1	3'-ggcatctcctcatcttcatcttct-5'	3'-cctaacttggctgctgtcttgg-5'

TABLE 3. List of MC/MC Transitions Used for Detection of Test Drug Biotransformation Products

Compound	m/z of the precursor	Fragment	Collision energy, eV
Warfarin	309.05	163.00	-15
Dasatinib	488.00	401.00	-30
7-Hydroxywarfarin	325.05	178.75	-20
4'-Hydroxydasatinib	504.00	416.88	-30

concentrations of testosterone, 6-hydroxybupropion, 4-hydroxytolbutamide, and 3-hydroxyomeprazole in the studied media were also measured.

Separation was carried out on a ZORBAX Eclipse Plus C18 HPLC column (4.6×150 mm, 5 μ) (Agilent) at a flow rate of 1 ml/min, 40°C, and the following gradient protocol: min 0-3 — 100% phase A (0.425 vol% formic acid in 5% acetonitrile solution in water), min 3-11 — linear gradient from 100 to 60% phase A, min 11-13 — linear gradient from 60 to 0% phase A, min 13-15 — 0% phase A, min 15-18 — linear gradient from 0 to 100% phase B, and min 18-21 — 100% phase B (0.425 vol% formic acid in acetonitrile).

Biotransformation products were detected on an LCMS 8030 chromatograph/mass-spectrometer (Shimadzu). Linear relationship was observed at 2-20 μM concentrations for 7-hydroxywarfarin and 0.5-5.0 μM for 4'-hydroxydasatinib (Table 3).

The data were statistically processed by Mann—Whitney nonparametric *U* test. The differences were considered significant at $p<0.05$.

RESULTS

Biotransformation of some drugs is realized with participation of various CYP450 isoforms and leads to the formation of two and more metabolites. If several drugs are used simultaneously, metabolism can be shifted towards the formation of an inert product. For example, biotransformation of clobazam leads to the formation of two metabolites: inert 4-hydroxyclobazam (metabolized with participation of CYP2C18 and CYP2C19) and active N-desmethylclobazam (metabolized with participation of CYP3A4, CYP2B6, and CYP2C19). Interaction of this drug with etravirin (drug with inhibitory activity towards CYP2C9 and CYP2C19) led to accumulation of active clobazam and to neurotoxic effects.

Simultaneous administration of dasatinib and warfarin is fraught with a high risk of hemorrhages [8].

Dasatinib is an antitumor drug with target activity, an inhibitor of tyrosine kinase. In nanomolar concentrations, the drug inhibits tyrosine kinases BCR-ABNL, SRC family (SRC, LCK, YES, and FYN), c-kit, EPHA2, and PDGFR and is used in the treatment of chronic myeloid leukemia in cases when imatinib cannot be used, and for Philadelphia chromosome-positive acute lymphoid leukemia (Ph+). It is also highly effective in metastatic melanoma. Dasatinib was created as a more effective imatinib analogue (*in vitro* dasatinib is 325 times more active than imatinib and 16 times more active than nilotinib) for the treatment of chronic leukemia. Simulation studies have shown that dasatinib binds many ABL kinase isoforms.

Warfarin is an indirect action anticoagulant. It is a racemate mixture of two active enanthomeres excreted from the body via different routes. S-warfarin is 5-fold more potent vitamin K antagonist than R-isomer. Warfarin is prescribed to patients with atrial fibrillation, artificial heart valves, thrombosis of deep veins, and antiphospholipid syndrome. Sometimes the drug is prescribed after myocardial infarction, but it is far less effective for prevention of new thromboses in the coronary arteries. For prevention of intra-arterial clotting, warfarin is administered in combination with anti-platelet drugs (*e.g.*, aspirin, clopidogrel) with the mechanisms of action different from those of warfarin (which is usually inessential for platelet function). Warfarin activity is partially determined by genetic factors, such as *VKORC1* and *CYP2C9* gene polymorphisms.

R-warfarin is metabolized with participation of CYP1A2 yielding 6-hydroxywarfarin and 8-hydroxywarfarin and with participation of CYP3A4 with the formation of 10-hydroxywarfarin (Fig. 1). The main warfarin metabolite is 7-hydroxywarfarin, a product of S-hydroxywarfarin biotransformation under the effect of CYP2C9. Drug-drug interactions with many com-

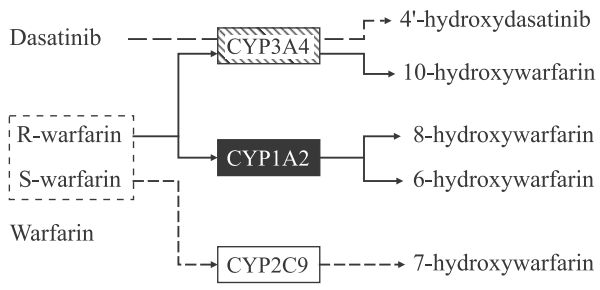

Fig. 1. Dasatinib and warfarin biotransformation catalyzed by CYP450. Continuous line: main route of metabolism; intermittent line: secondary route.

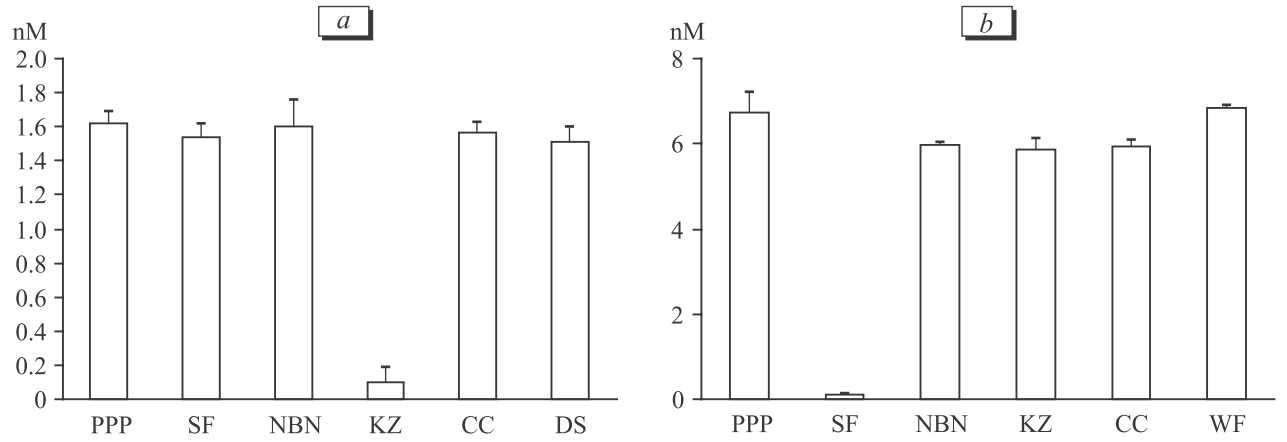

Fig. 2. Relationship between inhibitors of some CYP450 isoforms and the concentrations of the main metabolites of dasatinib — 4'-hydroxydasatinib (*a*) and warfarin — 7-hydroxywarfarin (*b*) in human liver cell model cultures. PPP: (2-phenyl-2-(1-piperdinil)-propane (CYP2B6B specific inhibitor); SF: sulfafenazole (CYP2C9 inhibitor); NBN: (+)-N-3-benzyl-nirvanol (CYP2C19); KZ: ketoconazole (CYP3A4). Control: SM: substrate mixture without inhibitors; DS: dasatinib, WF: warfarin without substrate mixture.

pounds metabolized by these CYP450 isoforms have been noted. Dasatinib biotransformation is realized with participation of CYP3A4 and with the formation of 4'-hydroxydasatinib (Fig. 1).

Mouse and human isoforms CYP3A and CYP2C are characterized by different substrate specificity [4]. The results of model studies of dasatinib-warfarin interactions cannot be extrapolated to humans, and hence, we selected human liver cell model (HLCM), HepaRG cell spheroids, for studies of biotransformation of these compounds.

First, we evaluated gene expression of CYP450 isoforms catalyzing biotransformation of the above drugs, CYP3A4 and CYP2C9, towards the reference genes *RPL23A*, *RAB10*, and *UBE2Q1*. The levels of *CYP3A4* and *CYP2C9* genes expression was 4- and 3-fold higher, respectively, than of the reference genes (data not presented).

After analysis of CYP450 isoform expression, the studied compounds were added to HepaRG cell culture with and without inhibitors and specific substrates. After 24-h co-culturing, the medium was sampled and metabolites were assayed. Sulfafenazole was an inhibitor of warfarin biotransformation, ketoconazole served as dasatinib inhibitor (Fig. 2). This indicated that warfarin biotransformation was realized with participation of CYP2C9 isoform, which is in line with previous data [7], while dasatinib biotransformation was realized with participation of CYP3A4, which was also in line with a previous report [6].

Simultaneous use of warfarin and dasatinib can lead to warfarin accumulation in the plasma. In addition, interactions with other drugs metabolized by CYP3A4 are probable [6].

Both warfarin and dasatinib inhibit tolbutamide and omeprazole biotransformations and are inessential for testosterone and bupropion biotransformations (Fig. 3). Hence, it seems that simultaneous use of these drugs with the compounds metabolized by CYP2C19 can lead to drug—drug interactions. As for dasatinib, it is undesirable to use it together with the drugs metabolized by CYP2C9, which is also in line with the previous report [15].

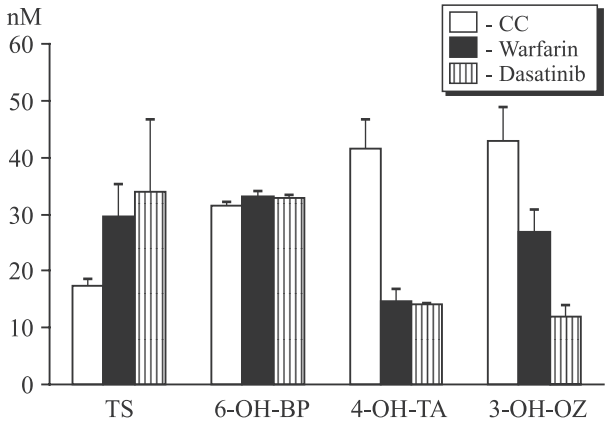

Fig. 3. Concentrations of substrates (testosterone — TS) and metabolites of 6-oxybupropion (6-OH-BP), 4-hydroxytolbutamide (4-OH-TA), 3-hydroxyomeprazole (3-OH-OZ) after 24-h incubation of the panel with 1 μM warfarin or dasatinib. The concentration of 3-hydroxyomeprazole is shown 100-fold higher than the actual one. SM: substrate mixture free from additives (control).

CYP450 substrate—inhibitor panel used in our study can be supplemented with new substrate—inhibitor combinations for more comprehensive reflection of the entire spectrum of CYP450 isoenzymes.

Importantly, recent studies indicate a special role of CYP450 isoforms in glucose metabolism. It is shown that inhibition of CYP7A1 in inflammation leads to accumulation of mevalonate pathway metabolites and hyperglycemia [14]. The use of our liver-

on-a chip model and specific substrates is expected to promote studies of the drug biotransformation and toxicity and their effects on glucose homeostasis during co-culturing of macrophages and human liver cell model under conditions of the nutrient medium microcirculation.

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