Pharmacological Reports 70 (2018) 972–980

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

Drug-drug interaction potential of antitumor acridine agent C-1748: The substrate of UDP-glucuronosyltransferases 2B7, 2B17 and the inhibitor of 1A9 and 2B7

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ARTICLE INFO

Article history:
Received 7 August 2017
Received in revised form 14 March 2018
Accepted 21 March 2018
Available online 22 March 2018

Keywords:
Antitumor agent C-1748
Drug-drug interactions
II phase metabolism
Glucuronidation
Enzyme inhibition
UGT

ABSTRACT

Background: The compound 9-(2′-hydroxyethylamino)-4-methyl-1-nitroacridine (C-1748), the promising antitumor agent developed in our laboratory was determined to undergo phase I metabolic pathways. The present studies aimed to know its biotransformation with phase II enzymes – UDP-glucuronosyltransferases (UGTs) and its potential to be engaged in drug-drug interactions arising from the modulation of UGT activity.

Methods: UGT-mediated transformations with rat liver (RLM), human liver (HLM), and human intestine (HIM) microsomes and with 10 recombinant human isoenzymes were investigated. Studies on the ability of C-1748 to inhibit UGT were performed with HLM, HT29 colorectal cancer cell homogenate and the selected recombinant UGT isoenzymes. The reactions were monitored using HPLC-UV/Vis method and the C-1748 metabolite structure was determined with ESI-TOF-MS/MS analysis.

Results: Pseudo-molecular ion (*m/z* 474.1554) and the experiment with β-glucuronidase indicated that *O*-glucuronide of C-1748 was formed in the presence of microsomal fractions. This reaction was selectively catalyzed by UGT2B7 and 2B17. High inhibitory effect of C-1748 was shown towards isoenzyme UGT1A9 (IC_{50} = 39.7 μM) and significant but low inhibitory potential was expressed in HT29 cell homogenate (IC_{50} = 84.5 μM). The mixed-type inhibition mechanism (K_i = 17.0 μM; K_i' = 81.0 μM), induced by C-1748 was observed for recombinant UGT1A9 glucuronidation, whereas HT29 cell homogenate resulted in noncompetitive inhibition (K_i = 94.6 μM).

Conclusions: The observed UGT-mediated metabolism of C-1748 and its ability to inhibit UGT activity should be considered as the potency for drug resistance and drug-drug interactions in the prospective multidrug therapy.

Introduction

UDP-glucuronosyltransferases (UGTs) catalyze the glucuronidation reaction of many endogenous and exogenous compounds leading to the metabolites more polar than aglycones, which can be readily excreted outside the body [1]. To date, at least 22 human UGT isoenzymes have been identified, which were classified into four gene families, UGT1, UGT2, UGT3 and UGT8 [2]. The individual UGT isoenzymes exhibit distinct, but overlapping, patterns of the substrates and inhibitor selectivities [3].

For a drug that undergoes UGT-mediated biotransformation, simultaneous administration of medications and dietary supplements can influence the drug metabolism by the inhibition of enzymes activity. UGT inhibition is regarded as one of the most important factor for clinical drug-drug and herb-drug interactions (DDIs, HDI) [4]. Common modulation effect on UGT activity is due to the fact that glucuronidation accounts for approximately 35% of phase II drug metabolism reactions [1].

It was shown that UGT-based DDIs were engaged in antidiabetic rapaglinide metabolism impairment during hypolipidemic gemfibrozil coadministration [5]. The nonsteroidal anti-inflammatory drug niflumic acid, being the substrate for UGT1A1 and to a lesser extent for several other UGTs is capable of inhibiting both UGT1A9 and UGT2B7 [6]. Miners et al. demonstrated that niflumic acid inhibited propofol and acetaminophen glucuronidation by HLM and recombinant isoenzymes [7]. Antitumor kinase inhibitors such as sorafenib, erlotinib and gefitinib were shown to inhibit UGT1A1 [8]. As a result the significant increase in the

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https://doi.org/10.1016/j.pharep.2018.03.007

exposure to irinotecan and its active metabolite SN-38 was observed [9]. Sorafenib exhibited also strong inhibition towards microsomal UGT1A9 [10].

Recently, many reports related to UGT-based HDIs have been published. For example licorice flavonoid licochalcone A exhibited a broad spectrum of inhibition against most of the human UGTs [11], whereas lactone compounds from *Andrographis paniculata* specifically inhibited UGT2B7 isoenzyme [12]. Specific stereoselective inhibitory effect on most UGT isoenzymes was demonstrated for triterpenoid saponins found in ginseng [13].

The studied here compound 9-(2′-hydroxyethylamino)-4-methyl-1-nitroacridine, C-1748 is one of the most promising antitumor agents developed in our laboratory. It exhibited strong cytotoxic activity towards colon cancer cell lines [14] as well as high antitumor activity against prostate carcinoma xenografts in nude mice [15]. Animal toxicity studies revealed C-1748 to have very low systemic toxicity, which allowed its selection for preclinical studies [16,17]. We showed earlier that C-1748 underwent phase I metabolism with human liver microsomes, human recombinant cytochrome P450 reductase (CPR) and in HepG2 cell line with the crucial role of hypoxic conditions. Metabolites identified as 1-aminoacridine derivatives and this one with an additional 6-membered ring were found. The key role of CPR, not cytochrome P450 3A4, in the activation mechanism of C-1748, was demonstrated recently. However, the overexpression of both CPR and P450 3A4 changed the proapoptotic activity and sensitized pancreatic cancer cells AsPC-1 to this drug [18,19].

Our current research is focused on phase II metabolism of C-1748 UGT, as well as to investigate the effect of this compound on UGT activity. Metabolism of C-1748 catalyzed by UGT in microsomal fractions and with human recombinant UGT isoenzymes was investigated and the structure of C-1748 glucuronide metabolite was identified. The effect of the drug on UGT activity was studied with three enzymatic systems: microsomal fraction, HT29 cell homogenate, and the selected human recombinant UGT isoenzymes.

Materials and methods

Chemicals and reagents

C-1748 9-(2′-hydroxyethylamino)-4-methyl-1-nitroacridine and C-857 9-(2′-hydroxyethylamino)-1-nitroacridine were synthesised according to the method described in Ref [20] 7-ethyl-10-hydroxycamptothecin (SN-38), 7-hydroxy-4-trifluoromethylcoumarin (HFC), 7-hydroxycoumarin, alamethicin 5 mg/ml in DMSO from *Trichoderma viride*, ammonium formate, dimethyl sulfoxide (DMSO), epirubicin hydrochloride, formic acid, magnesium chloride, trifluoperazine dihydrochloride (TFP), Tris hydrochloride, uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Disodium hydrogen phosphate, potassium chloride, potassium dihydrogen phosphate, sodium chloride were obtained from POCH S.A. (Gliwice, Poland). Methanol of HPLC grade was provided by Merck (KGaA, Darmstad, Germany).

Enzymes

Pooled microsomal fractions of: rat liver, 20 mg/ml (RLM), human liver, 20 mg/ml (HLM) and human intestine, 10 mg/ml (HIM) were purchased from Tebu-bio SAS (France). UGT Supersomes, 5 mg/ml (UGT1A1, 1A3, 1A4, 1A6, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17) were purchased from Corning (New York, USA). β-glucuronidase (GUS) type VII-A from *Escherichia coli*, 5,000,000–20,000,000 units/g protein was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and homogenate preparation

Human colorectal adenocarcinoma cell line HT29 was obtained from D. Banerjee from Department of Medicine and Pharmacology, Cancer Institute of New Jersey, USA. The cells were maintained as described previously [14]. Confluent cells were trypsinized with Trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged (1000 rpm, 5 min, 4 °C). The pellet was washed two times with phosphate-buffered saline (pH 7.4). The cell suspension was subjected to homogenization for 5 min in glass tissue grinder. Total cell homogenate protein concentration (28 mg/ml) was determined using the Bradford assay (Bio-Rad, USA).

Reactions with microsomal and recombinant UGTs

Screening for glucuronidation activity towards C-1748 was conducted with RLM, HLM, HIM and 10 recombinant human UGT isoenzymes. The proteins at a final concentration of 1 mg/ml were incubated with 0.1 mM C-1748 (20 mM stock solution in DMSO diluted with water) in a buffer containing 50 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$ and 25 μg/ml alamethicin. After preincubation for 5 min at 37 °C, UDPGA in water (final concentration of 5 mM) was added to initiate the reaction. The metabolism process was maintained at 37 °C for incubation times: 0/10/30/60/90/120/180/240 min and 0/30/60/120/240 min in the case of microsomal fractions and recombinant UGT isoenzymes, respectively. For kinetic analyses of C-1748 glucuronidation in HLM and HIM the substrate concentrations were: 0.01/0.025/0.05/0.1/0.25 mM. Other reaction mixture components concentrations and reaction conditions were as described above with the incubation time set at 60 min. The reactions were stopped by adding an equal volume (50 μl) of ice-cold methanol, which contained 0.025 mM C-857 as an internal standard in the case of microsomal fractions. In order to remove the proteins, the samples were centrifuged (13,400 rpm, 10 min). The supernatant fractions were used for HPLC analysis.

β-glucuronidase assay

Hydrolysis with β-glucuronidase (GUS) was used to identify the glucuronide peak. After incubation of 0.1 mM C-1748 with 0.5 mg/ml RLM and 5 mM UDPGA for 30 min, 1000 U of GUS was added, and the sample was incubated for the following 90 min. The reaction was performed and prepared for HPLC analysis as described above for microsomal and recombinant UGTs.

Investigation of UGT inhibition by C-1748

HFC, a nonselective substrate for UGT, was used as a probe substrate for HLM, UGT1A9, UGT1A10 and HT29 cells homogenate. For UGT1A1, UGT1A4 and UGT2B7 selective substrates: SN-38, TFP and epirubicin were used, respectively. All stock solutions (20 mM) were prepared in DMSO. The reactions of standard substrates in the absence or presence of C-1748 were performed and prepared for HPLC analysis as described above for microsomal and recombinant UGTs. The reaction conditions are summarized in Table 1.

Inhibition kinetic assays

Inhibition parameters: IC_{50} (concentration of inhibitor that reduces enzyme activity by 50%) and K_i/K_i' values (inhibition constants) were investigated for the glucuronidation of HFC by recombinant UGT1A9 and HT29 cell homogenate. To determine IC_{50} value, the glucuronidation rate was measured at fixed concentration of HFC – 0.1 mM. The concentrations of UGT1A9/HT29 homogenate was 0.2/0.5 mg/ml and UDPGA – 2 mM.

Table 1
Reaction conditions for the investigation of C-1748 inhibition potential towards UGTs.

Enzymatic fraction (conc., mg/ml)	Standard substrate (conc., mM)	UDPGA (conc., mM)	C-1748 (conc., mM)	Internal standard (conc., mM)	Incubation times (min)
HLM (0.1)	HFC (0.1)	0.2	0/0.01/0.1/0.2/0.5	7-hydroxycoumarin (0.01)	0, 10, 20, 30
UGT1A1 (1)	SN-38 (0.01)	5	0/0.001/0.01/0.1	C-857 (0.1)	0, 10, 30, 60, 90
UGT1A4 (1)	TFP (0.05)	5	0/0.01/0.05/0.1	7-hydroxycoumarin (0.01)	0, 30, 60, 90
UGT1A9 (0.5)	HFC (0.1)	5	0/0.01/0.1/0.2	7-hydroxycoumarin (0.01)	0, 10, 30, 60
UGT1A10 (0.5)	HFC (0.1)	5	0/0.01/0.1/0.2	7-hydroxycoumarin (0.01)	0, 30, 60, 90
UGT2B7 (1)	epirubicin (0.1)	5	0/0.01/0.1/0.2	C-857 (0.1)	0, 30, 60, 90
HT29 homogenate (0.5)	HFC (0.1)	2	0/0.01/0.1/0.2	7-hydroxycoumarin (0.01)	0, 10, 30, 60, 90

The incubation times were 20 and 30 min for UGT1A9 and HT29 homogenate, respectively. The inhibitor concentrations were 0/0.025/0.05/0.1/0.25/0.5 mM. K_i and K_i' values were determined by using various concentrations of HFC: 0.025/0.05/0.1/0.25 mM. Other reaction mixture components concentrations and incubation times were the same as described above. The reaction was performed and prepared for HPLC analysis as described above for microsomal and recombinant UGTs. 7-Hydroxycoumarin (0.01 mM) was used as an internal standard.

HPLC-UV/Vis analysis

Aliquots (50 μl) of the incubation mixtures were analyzed by reversed-phase HPLC method with UV/Vis detection as described previously [21]. Five μm Suplex pKb-100 analytical column (0.46 cm x 25 cm, C18) (SUPELCO, Bellefonte, PA, USA) with Agilent 1100 (Agilent Technologies, Santa Clara, CA, USA) HPLC system were applied. Analyses were performed at a flow rate of 1 ml/min using two eluents: A – ammonium formate (0.05 M, pH 3.4) +5% v/v methanol and B – methanol +5% v/v water. The elution programme and detection wavelength depended on the analyte:

- C-1748 and its metabolite (430 nm), SN-38 (380 nm), epirubicin (480 nm): a linear gradient 15–80% eluent B in eluent A for 25 min, followed by linear gradient 80–100% eluent B in eluent A for 3 min;
- TFP (303 nm): a linear gradient 40–100% eluent B in eluent A for 30 min
- HFC (330 nm): a linear gradient 30–80% eluent B in eluent A for 20 min, followed by linear gradient 80–100% eluent B in eluent A for 5 min.

ESI–MS analysis of the metabolite. Reaction mixtures (20 μl) obtained after 60 min incubation of 0.1 mM C-1748 with 1 mg/ml RLM and 5 mM UDPGA, were analyzed by HPLC–MS and HPLC–MS/MS spectra of the substrate and metabolite after electrospray ionisation (ESI) with positive ion detection in the range *m/z* 100–700. Nitrogen was used as the nebulizer, desolvation and cone gas. MS conditions: gas temperature 325 °C, flow rate 10 l/min, gas pressure 35 psi, and capillary and fragmentor potentials 3500 and 175 V, respectively [19].

Data analysis

The results are presented as mean ± SD of at least two independent experiments. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, USA). One-way analysis of variance (ANOVA) followed by Bonferroni test was applied to compare the differences between groups; $^{*}p < 0.05/^{**}p < 0.01/^{***}p < 0.001$ were considered significant.

The quantitation of the C-1748 glucuronide formed by HLM and HIM was achieved by calibrating with the substrate as the glucuronidation does not change the UV–vis spectra of C-1748. Kinetic parameters of C-1748 glucuronidation: K_m (Michaelis-Menten constant) and V_{max} (maximum velocity) as well as inhibition parameters: IC_{50} and K_i/K_i' values (Ki: dissociation constant of enzyme-inhibitor complex and Ki': of enzyme-substrate-inhibitor complex), were determined by fitting the experimental data to nonlinear regression model using GraphPad Prism 5 software.

Results

UGT-mediated metabolism of C-1748 with microsomal fractions

Incubations of C-1748 with human liver (HLM) and human intestine (HIM) microsomes in the presence of UDPGA led to one identical metabolite, M (Fig. 1A), as indicated the UV–vis spectra. This metabolite, characterized by a slightly longer, than the substrate, retention time was observed in HLM at higher concentration than in HIM. The Michaelis-Menten curves of the metabolite formation (Fig. 1B) and the kinetic parameters present in Table 2 indicated 2-fold higher catalytic rate of HLM than HIM, whereas K_m values were 3 fold higher for HLM in comparison to that for HIM. Therefore, the results indicated that the substrate affinity was almost 3-fold higher for HIM than HLM, whereas the calculated intrinsic clearance (CL_{int}) seemed to be comparable in HLM and HIM with only slight tendency to be higher in HIM.

Identification of C-1748 metabolite structure

Rat liver microsomes (RLM) were applied to identify the metabolite structure, because much higher metabolite concentration was observed in this case (Fig. 2A). The analysis of the reaction mixture containing C-1748 and its metabolite M after the following β-glucuronidase action showed only the substrate C-1748 (Fig. 2A). Therefore, the hydrolysis of the metabolite occurred, what indicated that glucuronide derivative of C-1748 was obtained with microsomal fractions. To confirm this indication we performed ESI-TOF-MS experiments with the reaction mixtures containing C-1748 and its metabolite. The protonated molecular ions $[M+H]^+$ of C-1748 and M metabolite were present at *m/z* 298.1212 and 474.1554, respectively, indicating characteristic mass difference for glucuronic acid (176 Da). MS/MS spectrum of metabolite M (Fig. 2B) illustrates the fragmentation giving *m/z* 298.1193, which was equal to that of C-1748 mass units. This supports the presence of glucuronide in the reaction mixture. The next question was whether *N*- or *O*-glucuronidation occurred. Reaction scheme (Fig. 2C) presents the structure of the proposed product as *O*-glucuronide on the C-1748 side chain, because the glucuronidation of an aromatic 9-amino group should give the changes in the metabolite UV–vis spectrum, which was not observed for C-1748 glucuronide. Moreover, we have not observed the glucuronidation product of

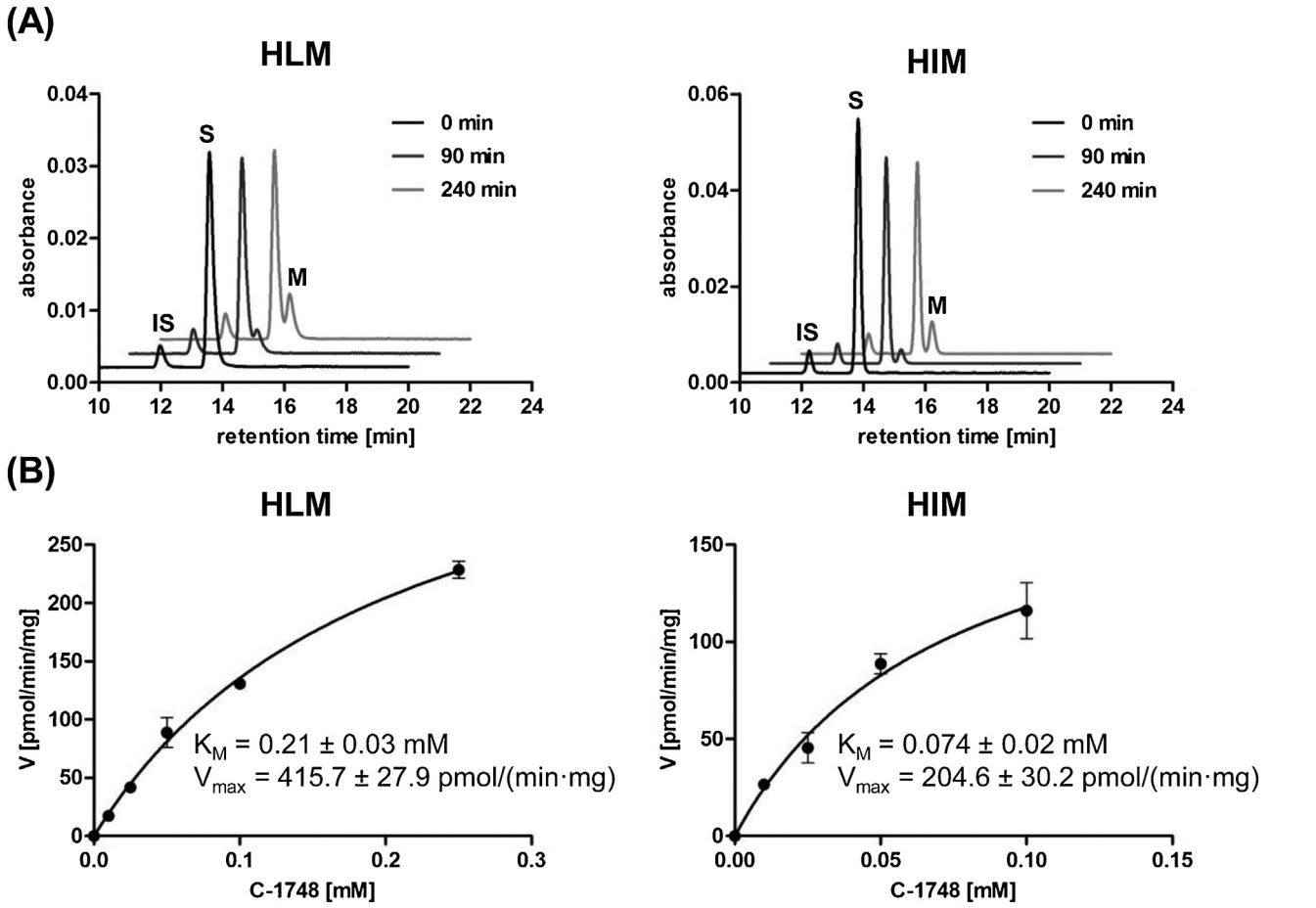

Fig. 1. Glucuronidation of C-1748 in human liver microsomes (HLM) and human intestine microsomes (HIM). (A) C-1748 metabolite formation in the presence of microsomal fractions and UDPGA. The representative chromatograms of the reaction mixtures after selected incubation times. (B) Michaelis-Menten kinetics of C-1748 glucuronidation in HLM and HIM. IS – internal standard, S – substrate, M – metabolite.

C-1748 analog, which possess the amino- instead of the hydroxyl group in the side chain (data not shown). This result indicated that N-glucuronidation did not occur and confirmed that only oxygen atom at C-1748 side chain is able to be glucuronidated.

Human recombinant UGT isoforms involved in C-1748 metabolism

The panel of human recombinant UGT isoenzymes: 1A1, 1A3, 1A4, 1A6, 1A9, 1A10 and 2B4, 2B7, 2B15, 2B17 was applied to test the glucuronidation susceptibility of C-1748. All tested isoenzymes of the UGT1A subfamily did not metabolize this compound (data not shown). Only UGT2B7 and UGT2B17 among UGT2 B family exhibited slight activity towards C-1748. HPLC chromatograms (data not shown) indicate the presence of the metabolite with the characteristic identical to that obtained for metabolite M with microsomal fractions (Figs. 1A, 2A) and described above as *O*-glucuronide of C-1748 (Fig. 2C).

Inhibitory potential of C-1748 towards glucuronidation (HLM, HT29 cells, UGT isoenzymes)

The effect of C-1748 on UGTs' activity was investigated with a nonselective substrate HFC in two model systems: HLM and HT29 colon cancer cell line homogenate. HT29 cell line was selected due to high activity of C-1748 observed against this cell line [14] and because of relatively high UGT expression level [22]. The conversion rate of HFC was assessed in the absence or presence of C-1748 at various concentrations (0.01, 0.1, 0.2 mM for both HLM and HT29 homogenate and one more, 0.5 mM for HLM). Results indicated that C-1748 inhibited the microsomal glucuronidation only at high concentration 0.5 mM, whereas 0.1 and 0.2 mM of this compound were sufficient to reduce the glucuronidation rate in HT29 homogenate (Fig. 3A). Despite being statistically significant, this rate reduction was rather low, not higher than 10%, and 20% for concentrations 0.1 and 0.2 mM, respectively.

Table 2
Enzyme kinetic parameters of C-1748 metabolite formation with HLM and HIM.

Microsomal fraction	Parameter		
	V_{max} [pmol/(min mg protein)]	K_M [mM]	CL_{int} [ml/(min mg protein)]
HLM	415.7 ± 27.9	0.21 ± 0.03	1.98
HIM	204.6 ± 30.2	0.074 ± 0.02	2.76

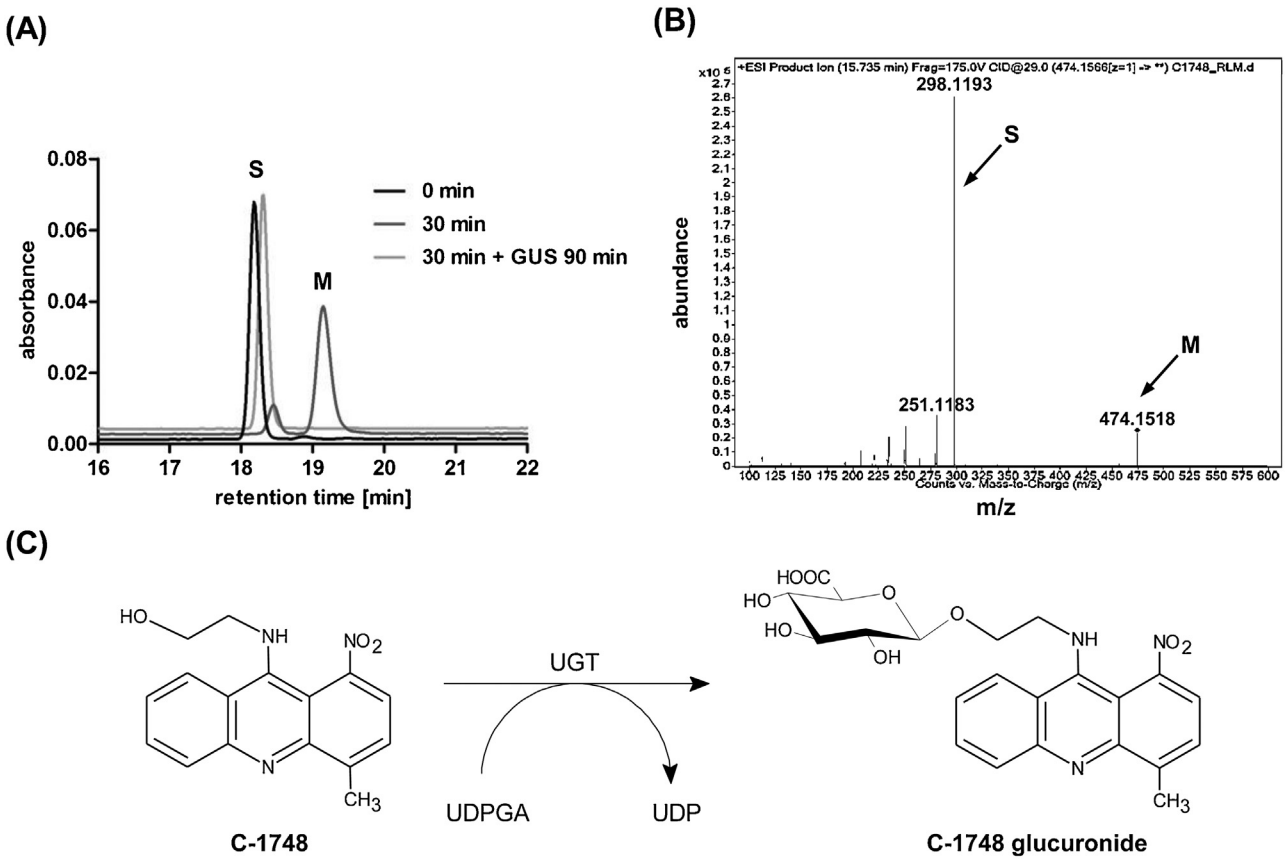

Fig. 2. Identification of C-1748 metabolite observed in the presence of rat liver microsomes (RLM) and UDPGA. (A) HPLC-UV/Vis analysis of the reaction mixtures after incubation of C-1748 with RLM and the following incubation with β-glucuronidase; (B) ESI-TOF-MS/MS spectrum of C-1748 metabolite; (C) Scheme of the reaction proposed for the glucuronidation of C-1748. GUS – β-glucuronidase, $[C–1748 + H]^+$ – substrate, $[M+H]^+$ – metabolite.

Inhibition of human recombinant UGT isoenzymes by C-1748 was assessed with the standard substrates (SN-38–UGT1A1, TFP – UGT1A4, HFC – UGT1A9/1A10 and epirubicin – UGT2B7) in the absence or presence of C-1748 at several concentrations. Various impact of C-1748 on the glucuronidation activity for each UGT isoenzyme was demonstrated (Fig. 3B). No statistically significant effect of the drug on UGT1A1, 1A4 and 1A10 action was found. However, UGT1A9 and UGT2B7 gave approximately 50% and 40% inhibition, respectively, at the longest incubation time with 0.1 mM C-1748.

UGT inhibition kinetics for HT29 cells and UGT1A9 isoenzyme by C-1748

Firstly, the IC_{50} values of C-1748 for HFC glucuronidation catalyzed by HT29 cell homogenate and human recombinant UGT1A9 isoenzyme were determined. Secondly, kinetic experiments were performed for further characterization of the inhibition induced by C-1748 in both enzymatic systems. HT29 cell homogenate and UGT1A9 isoenzyme were selected because their glucuronidation potentials were the most sensitive to the inhibitory effect of C-1748 (Fig. 3). IC_{50} values determined from the inhibition curves (Fig. 4A) were 84.5 and 39.7 μM for HT29 homogenate and UGT1A9, respectively. Kinetics of HFC glucuronidation in the presence of various concentrations of C-1748 obtained by fitting the data to nonlinear regression model (Fig. 4B) revealed that C-1748 inhibited HFC glucuronidation activity in HT29 cell homogenates and human recombinant UGT1A9 enzyme according to non-competitive and mixed mechanism, respectively. The estimated K_i value for inhibition of HFC glucuronidation activity in HT29 cell homogenates was 95 μM whereas K_i and K_i' values for inhibition of HFC glucuronidation activity in human recombinant UGT1A9 enzyme were 17 and 81 μM, respectively.

Discussion

Currently, two forms of resistance to anticancer chemotherapy have been proposed: intrinsic and acquired drug resistance. Intrinsic resistance is a pre-existing feature of tumor cells that is present prior to the drug exposure, whereas acquired resistance is developed after exposure of an initially sensitive tumor to the drug [23]. High level of drug-metabolizing enzymes, which are engaged in drug detoxification and the following drug excretion can be responsible for the intrinsic resistance in tumor cells. Glucuronidation as one of the major deactivation pathway of antitumor agents was reported to be strongly implicated in this type of resistance [24]. A therapeutic strategy based on the reversion of UGT-mediated intrinsic drug resistance by using selective UGT inhibitors has been proposed [22]. However, it is rather complex approach. On the one hand it can restore drug activity, but on the other hand, can impair the detoxification of other exogenous and endogenous UGT substrates. Therefore, the prediction of the optimal conditions for the balance between therapeutic effectiveness and adverse side-effects, both resulted from UGT-mediated metabolism, is very difficult, but crucial for the design of effective antitumor therapy.

Considering all above, the purpose of the present work was focused on two aspects. Firstly, we intended to determine the susceptibility of C-1748 antitumor agent to UGT-mediated metabolism in order to predict the possibility of intrinsic drug

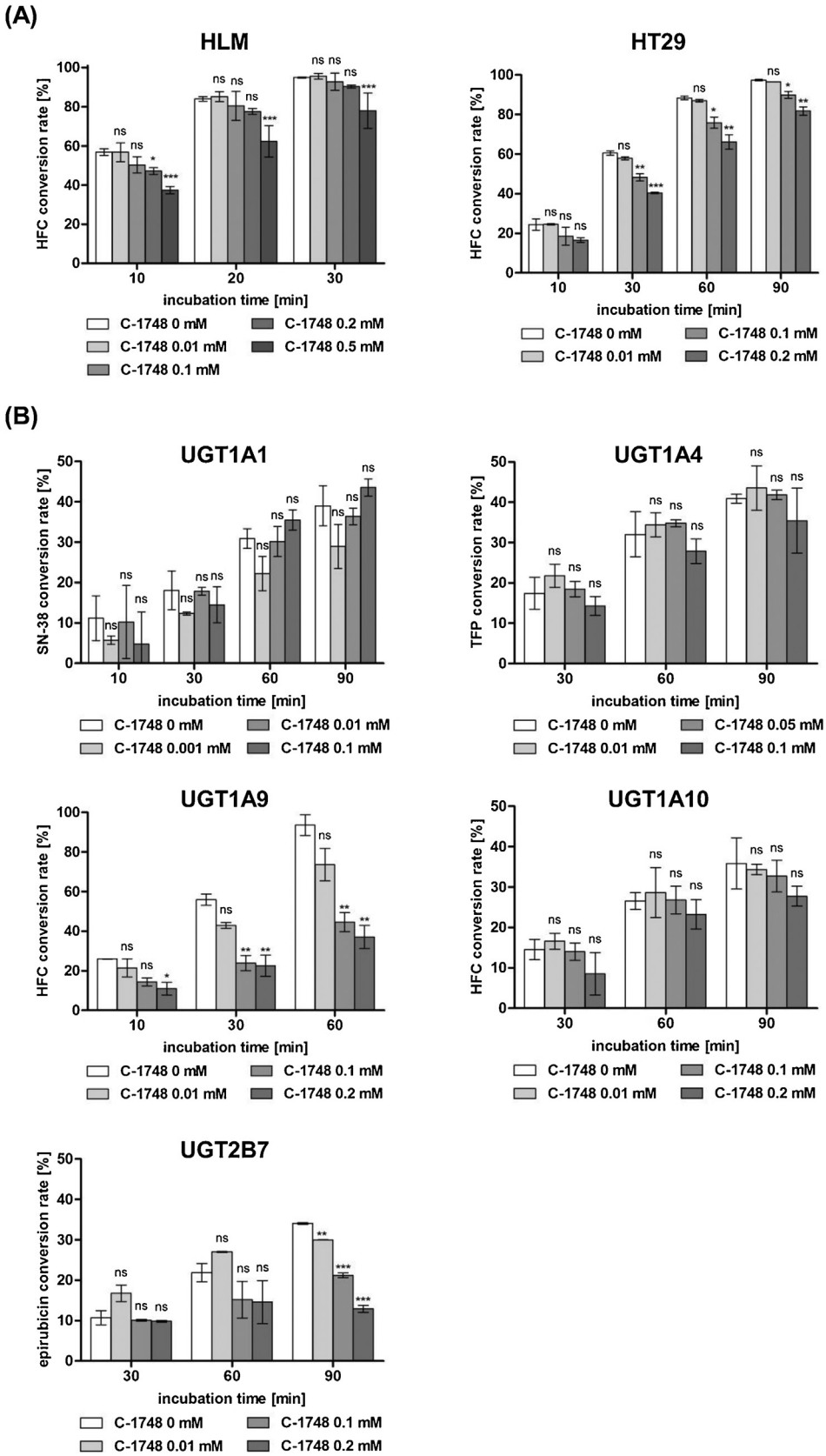

Fig. 3. The effect of C-1748 on UGT activity. The modulation of: (A) HFC glucuronidation with human liver microsomes (HLM) and HT29 cell homogenate; and (B) glucuronidation activity of human recombinant UGT isoenzymes studied with the specific substrates: SN-38 for UGT1A1, TFP for UGT1A4, HFC for UGT1A9/A10 and epirubicin for UGT2B7. Enzymatic activities towards standard substrates in the absence or presence of C-1748 are expressed as a conversion rate of the substrate after various incubation times. $^{*}p < 0.05/^{**}p < 0.01/^{***}p < 0.001$/ns – non-significant.

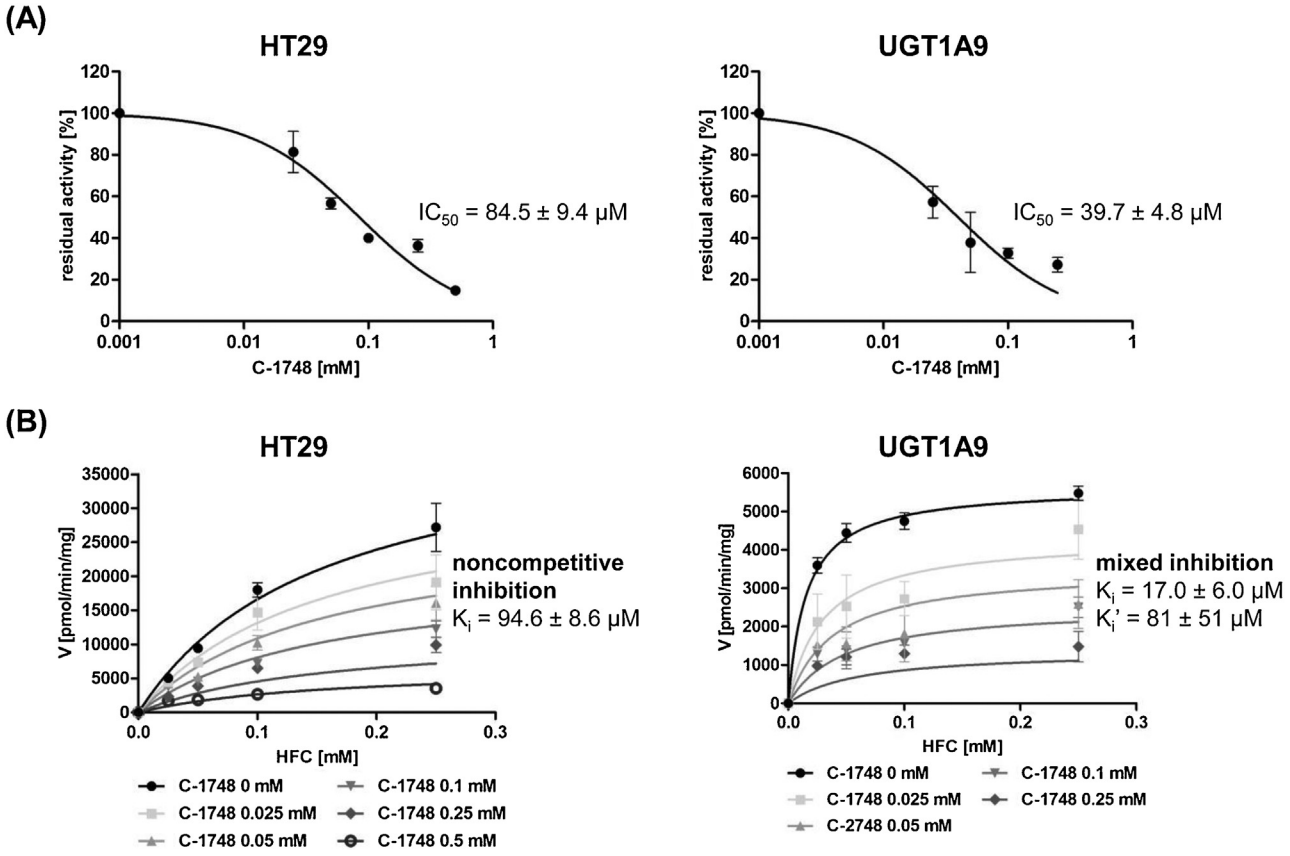

Fig. 4. The inhibitory effect of C-1748 on UGT activity. (A) Inhibitory effect of C-1748 on HFC glucuronidation activity in HT29 cell homogenates and human recombinant UGT1A9 isoenzyme with IC_{50} values; (B) Michaelis- Menten plots for the inhibitory effect of C-1748 on HFC glucuronidation activity in HT29 cell homogenates and human recombinant UGT1A9 enzyme. Data represent the mean ± SD.

resistance. Secondly, we aimed to investigate the influence of C-1748 on the UGTs activity in the framework of DDIs, which can be observed in multidrug therapy.

To predict the risk of C-1748 drug resistance we performed reactions of studied compound with microsomal fractions from human liver and human intestines (HLM, HIM) in the presence of UGT cofactor UDPGA. We observed metabolite formation in both cases and kinetic parameters K_m and V_{max} as well as the values of intrinsic clearance, CL_{int}, were determined. There was demonstrated that HLM were characterized by two times higher catalytic rate than HIM, whereas HIM showed nearly three times higher affinity towards studied compound than HLM. Therefore, the intrinsic clearance of this drug would be expected slightly higher with intestine than liver enzymes, whereas the majority of therapeutics undergo UDP-glucuronidation with liver enzymes [6,22].

Mass spectrometry analyses as well as β-glucuronidase assay indicated the formation of the *O*-glucuronide (Fig. 2C). Considering tissue-specific expression of UGT isoenzymes it was important to identify the isoforms responsible for drug glucuronidation in order to predict the drug effect in different organs [25]. The screening of a panel of UGT human recombinant isoenzymes for glucuronidation of C-1748 did not detect any metabolite in the presence of six UGT1A isoenzymes, whereas only two of UGT2B (2B7 and 2B17) catalyzed C-1748 glucuronidation. However, in both cases the concentrations of the obtained glucuronides were very low, what indicated only slight tendency of the studied acridine to deactivation by human UGT isoenzymes. Therefore, UGT-dependent intrinsic drug resistance would not be observed in the case of C-1748 giving the chance to keep the antitumor effect after this drug treatment. In order to explain why the studied compound is rather marginally metabolized by UGT isoenzymes we have to consider that the product of C-1748 glucuronidation was formed by the transfer of glucuronic acid on the aliphatic hydroxyl group of the side chain of acridine ring (Fig. 2C). In contrast, the UGT conjugation of aromatic hydroxyl groups usually occurs with much higher efficiency than aliphatic ones, as it was demonstrated for 17β-estradiol and morphine [26].

The studies on the inhibitory potential of antitumor agents towards UGTs activity should consider the role of UGT enzymes in metabolism of endogenous substrates such as bilirubin, bile acids, lipid acids, steroid and thyroid hormones. Another aspect is the glucuronidation of xenobiotics, including environmental pollutants and therapeutic agents [2]. There are also reports on the role of differences in UGT expression and/or activity in tumor *vs.* normal tissue. Thus, we can suppose that drug-induced changes in UGT level and activity may interfere with tumor cell growth as well as with the proliferation and homeostasis in normal cells [27].

The investigation of C-1748 influence on UGT activity demonstrated the significant, but low inhibitory effect of 0.1 mM C-1748 towards HT29 cell homogenate, whereas approximately 50% and 40% inhibitions were demonstrated for isoenzymes UGT1A9 and UGT2B7, respectively. The inhibition of HLM-mediated glucuronidation was rather negligible. The IC_{50} values of C-1748 towards glucuronidation activity of HT29 cell homogenate and human recombinant UGT1A9 were 84.5 and 39.7 μM, respectively. The comparison of these results with other published data allowed to classify the inhibitory potential of C-1748 as moderate (UGT1A9, UGT2B7) and almost negligible (HT29) [28,29].

The inhibition kinetic analysis resulted in rather high K_i value (95 μM) for HT29 cell homogenate, which indicated that the risk of DDIs is unlikely during treatment the cell with C-1748 [29]. However, lower K_i value (17 μM) found for recombinant UGT1A9 suggests that DDIs may occur when combining C-1748 with drugs selectively metabolized by UGT1A9. It is the only isoform that catalyzes the glucuronidation of anaesthetic agent propofol in the liver and the most active isoform towards immunosuppressant mycophenolic acid [30]. Besides, significant contribution of UGT1A9 to acetaminophen [31] and irinotecan metabolite, SN-38 [32] glucuronidation was shown.

We also observed the significant inhibitory potential of C-1748 towards UGT2B7, which is responsible for the biotransformation of 35% of drugs undergoing glucuronidation [33]. It should be assumed that the co-administration of C-1748 with drugs undergoing UGT2B7-mediated metabolism as antitumor epirubicin [34], opioids [35] and antiretroviral agent zidovudine [36] may result in DDIs. Recent studies revealed lower glucuronidation capacities of both UGT1A9 and 2B7 in kidney tumor tissue in relation to normal tissue [37]. Therefore, the inhibitory effect of C-1748 on these isoenzymes in tumor tissue should be taken into account because it would strongly diminish the detoxification of other therapeutics.

Summing up, our *in vitro* data demonstrated that the potent antitumor acridine agent, C-1748, underwent glucuronidation with microsomal UGTs. Enzymes of UGT1 family were not involved in metabolism of this drug, whereas isoenzymes UGT2B7 and 2B17 catalyzed this transformation. The significant inhibitory effect of C-1748 towards UGT-mediated metabolism in HT29 cells as well as with UGT1A9 and 2B7 recombinant isoenzymes was also demonstrated. Therefore, the selective influence of C-1748 on the pathways catalyzed only by isoenzymes UGT1A9 and UGT2B7 created the future potency of this drug. It would be able to inhibit selectively the glucuronidation of other antitumor drugs only in the case when they were metabolized selectively with UGT1A9 and UGT2B7 isoenzymes. The above conclusion is strongly helpful for the prediction of drug-drug interactions in respect to drug resistance in the prospective multidrug therapy. The obtained results provided the background for the further preclinical and clinical investigations of antitumor potency of C-1748 in respect to its UGT inhibition properties.

Conflict of interest

None.

Authors' contribution

Participated in research design: Mazerska, Mróz; conducted experiments: Mróz, Ryska, Sominko, Bejrowska; performed data analysis: Mróz, Bejrowska, Mazerska; wrote or contributed to the writing of the manuscript: Mróz, Mazerska.

All authors have approved the final article.

Acknowledgments

This work was supported by the National Science Center, Poland, grant OPUS5, No 2013/09/B/NZ3/00003 and by R&D grants No 031449/2015 and 031966/2016 from the Chemical Faculty of Gdańsk University of Technology, Poland.

We are grateful to Prof. Agata Kot-Wasik, Kamila Wilczewska and Paweł Kubica (Department of Analytical Chemistry) for their help with mass spectrometry analysis.

We are grateful to Ewa Augustin Dr.Sc. for fruitful discussions and support in cell experiments. Part of this work was presented as poster at 13th European ISSX Meeting, Glasgow, 2015; International Society for the Study of Xenobiotics, Abstract no. P19, p 50.

References

[1] Guillmette C.. Pharmacogenomics of human UDP-glucuronosyltransferase enzymes. Xenobiotica 2003;3:136–58.
[2] Rowland A, Miners JO, Mackenzie PI. The UDP-glucuronosyltransferases: their role in drug metabolism and detoxification. Int J Biochem Cell Biol 2013;45:1121–32.
[3] Miners JO, Mackenzie PI, Knights KM. The prediction of drug glucuronidation parameters in humans: UDP-glucuronosyltransferase enzyme selective substrate and inhibitor probes for reaction phenotyping and in vitro-in vivo extrapolation of drug clearance and DDIs potential. Drug Metab Rev 2010;42:196–208.
[4] Kiang TKL, Ensom MHH, Chang TKH. UDP-glucuronosyltransferases and clinical drug-drug interactions. Pharmacol Ther 2005;106:97–132.
[5] Gan J, Chen W, Shen H, Gao L, Hong Y, Tian Y, et al. Rapaglinide-gemfibrozil drug interaction: inhibition of rapaglinide glucuronidation as a potential additional contributing mechanism. Br J Clin Pharmacol 2010;70:870–80.
[6] Gaganis P, Miners JO, Knights KM. Glucuronidation of fenamates: kinetic studies using human kidney cortical microsomes and recombinant UDP-glucuronosyltransferase (UGT) 1A9 and 2B7. Biochem Pharmacol 2007;73:1683–91.
[7] Miners JO, Bowalgaha K, Elliot DJ, Baranczewski P. Characterization of niflumic acid as a selective inhibitor of human liver micarosomal UDP-glucuronosyltransferase 1A9: application to the reaction phenotyping of acetaminophen glucuronidation. Drug Metab Dispos 2011;39:644–52.
[8] Liu Y, Ramírez J, House L, Ratain MJ. Comparison of the drug-drug interactions potential of erlotinib and gefitinib via inhibition of UDP-glucuronosyltransferases. Drug Metab Dispos 2010;38:32–9.
[9] Mross K, Steinbild S, Baas F, Gmehling D, Radtke M, Voliotis D, et al. Results from an in vitro and clinical/pharmacological phase I study with combination irinotecan and sorafenib. Eur J Cancer 2007;43:55–63.
[10] Miners JO, Chau N, Rowland A, Burns K, McKinnon RA, Mackenzie PI, et al. Inhibition of human UDP-glucuronosyltransferase enzymes by lapatinib, pazopanib, regorafenib and sorafenib: implications for hyperbilirubinemia. Biochem Pharmacol 2017;129:85–95.
[11] Xin H, Qi XY, Wu JJ, Wang XX, Li Y, Hong JY, et al. Assessment of the inhibition potential of Licochalcone A against human UDP-glucuronosyltransferases. Food Chem Toxicol 2016;90:112–22.
[12] Ma HY, Sun DX, Cao YF, Ai CZ, Qu YQ, Hu CM, et al. Herb-drug interaction prediction based on the high specific inhibition of andrographolide derivatives towards UDP-glucuronosyltransferase (UGT) 2B7. Toxicol Appl Pharmacol 2014;277:86–94.
[13] Kim D, Zheng YF, Min JS, Park JB, Bae SH, Yoon KD, et al. In vitro stereoselective inhibition of ginsenosides toward UDP-glucuronosyltransferase (UGT) isoforms. Toxicol Lett 2016;259:1–10.
[14] Augustin E, Mośr-Rompa A, Nowak-Ziatyk D, Konopa J. Antitumor 1-nitroacridine derivative C-1748, induces apoptosis, necrosis or senescence in human colon carcinoma HCT8 and HT29 cells. Biochem Pharmacol 2010;79:1231–41.
[15] Tadi K, Ashok BT, Chen Y, Banerjee D, Wysocka-Skrzela B, Konopa J, et al. Pre-clinical evaluation of 1-nitroacridine derived chemotherapeutic agent that has preferential cytotoxic activity towards prostate cancer. Cancer Biol Ther 2007;6:1632–7.
[16] Ashok BT, Tadi K, Banerjee D, Konopa J, Iatropoulos M, Tiwari RK. Pre-clinical toxicology and pathology of 9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine (C-1748), a novel anti-cancer agent in male Beagle dogs. Life Sci 2006;79:1334–42.
[17] Ashok BT, Tadi K, Garikapaty VP, Chen Y, Huang Q, Banerjee D, et al. Preclinical toxicological examination of a putative prostate cancer-specific 4-methyl-1-nitroacridine derivative in rodents. Anticancer Drugs 2007;18:87–94.
[18] Wiśniewska A, Niemira M, Jagiełło K, Potęga A, Swist M, Henderson C, et al. Diminished toxicity of C-1748, 4-methyl-9-hydroxyethylamino-1-nitroacridine, compared with its demethyl analog, C-857, corresponds to its resistance to metabolism in HepG2 cells. Biochem Pharmacol 2012;84:30–42.
[19] Borowa-Mazgaj B, Mróz A, Augustin E, Paluszkiewicz E, Mazerska Z. The overexpression of CPR and P450 3A4 in pancreatic cancer cells changes the metabolic profile and increases the cytotoxicity and pro-apoptotic activity of acridine antitumor agent, C-1748. Biochem Pharmacol 2017;142:21–38.
[20] J. Konopa, B. Wysocka-Skrzela, R.K. Tiwari. 9-Alkilamino-1-nitroacridine derivatives, European patent, 01910914.9-2101-US0105199, American patent, 1981.
[21] Potęga A, Fedejko-Kap B, Mazerska Z. Mechanism-based inactivation of human cytochrome P450 1A2 and 3A4 isoenzymes by anti-tumor triazoloacridinone C-1305. Xenobiotica 2016;46:1056–65.
[22] Cummings J, Ethell BT, Jardine L, Boyd G, Macpherson JS, Burchell B, et al. Glucuronidation as a mechanism of intrinsic drug resistance in human colon cancer: reversal of resistance by food additives. Cancer Res 2003;63:8443–50.
[23] Lippert TH, Ruoff HJ, Volm M. Intrinsic and acquired drug resistance in malignant tumors: the main reason for therapeutic failure. Arzneimittelforschung 2008;58:261–4.
[24] Mazerska Z, Mróz A, Pawłowska M, Augustin E. The role of glucuronidation in drug resistance. Pharmacol Ther 2016;159:35–55.

[25] Court MH, Zhang X, Ding X, Yee KK, Hesse LM, Finel M. Quantitative distribution of mRNAs encoding the 19 human UDP-glucuronosyltransferase enzymes in 26 adult and 3 fetal tissues. Xenobiotica 2012;42:266–77.
[26] Parkinson A, Ogilvie BW, Paris BL, Hensley TN, Loewen GJ. Human biotransformation. In: Nasar AF, editor. Biotransformation and Metabolite Elucidation of Xenobiotics. Hoboken, New Jersey: John Wiley & Sons, Inc; 2010.
[27] Dates CR, Fahmi T, Pyrek SJ, Yao-Borengasser A, Borowa-Mazgaj B, Bratton SM, et al. Human UDP-glucuronosyltransferases: effects of altered expression in breast and pancreatic cancer cell lines. Cancer Biol Ther 2015;16:714–23.
[28] Joo J, Kim YW, Wu Z, Shin JH, Lee B, Shon JC, et al. Screening of non-steroidal anti-inflammatory drugs for inhibitory effects on the activities of six UDPglucuronosyltransferases (UGT1A1, 1A3, 1A4, 1A6, 1A9 and 2B7) using LC-MS/MS. Biopharm Drug Dispos 2015;36:258–64.
[29] Zhang N, Liu Y, Jeong H. Drug–Drug interaction potentials of tyrosine kinase inhibitors via inhibition of UDP-glucuronosyltransferases. Sci Rep 2015;5:17778.
[30] Court MH. Isoform-selective probe substrates for in vitro studies of human UDP-glucuronosyltransferases. Methods Enzymol 2005;400:104–16.
[31] Court MH, Duan SX, von Moltke LL, Greenblatt DJ, Patten CJ, Miners JO, et al. Interindividual variability in acetaminophen glucuronidation by human liver microsomes: identification of relevant acetaminophen UDP-glucuronosyltransferase isoforms. J Pharmacol Exp Ther 2001;299:998–1006.
[32] Hanioka N, Ozawa S, Jinno H, Ando M, Saito Y, Sawada J. Human liver UDP-glucuronosyltransferase isoforms involved in the glucuronidation of 7-ethyl-10-hydroxycamptothecin. Xenobiotica 2001;31:687–99.
[33] Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, et al. Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. Drug Metab Dispos 2004;32:120–8.
[34] Innocenti F, Iyer L, Ramírez J, Green MD, Ratain MJ. Epirubicin glucuronidation is catalyzed by human UDP-glucuronosyltransferase 2B7. Drug Metab Dispos 2001;29:686–92.
[35] Coffman BL, King CD, Rios GR, Tephly TR. The glucuronidation of opioids, other xenobiotics, and androgens by human UGT2B7Y(268) and UGT2B7H(268). Drug Metab Dispos 1998;26:73–7.
[36] Barbier O, Turgeon D, Girard C, Green MD. Tephly TR, Hum DW, Bélanger A. 3'-azido-3'-deoxythimidine (AZT) is glucuronidated by human UDP-glucuronosyltransferase 2B7 (UGT2B7). Drug Metab Dispos 2000;28:497–502.
[37] Margaillan G, Rouleau M, Fallon JK, Caron P, Villeneuve L, Turcotte V. et al: quantitative profiling of human renal UDP-glucuronosyltransferases and glucuronidation activity: a comparison of normal and tumoral kidney tissues. Drug Metab Dispos 2015;43:611–9.