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Regular article

Comparative metabolic study between two selective estrogen receptor modulators, toremifene and tamoxifen, in human liver microsomes☆

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ABSTRACT

Toremifene (TOR) and Tamoxifen (TAM) are widely used as endocrine therapy for estrogen receptor positive breast cancer. Poor metabolizers of TAM are likely to have worse clinical outcomes than patients who exhibit normal TAM metabolism due to lower plasma level of its active metabolite, 4-hydroxy-*N*-desmethyl (4OH-NDM) tamoxifen (endoxifen). In this study, we examined the role of individual cytochrome P450 (CYP) isoforms in the metabolism of TOR to *N*-desmethyl (NDM), 4-hydroxy (4OH) and 4OH-NDM metabolites in comparison with TAM using human liver microsomes (HLMs) with selective chemical inhibitors for each CYP isoform and recombinant CYP proteins. Similar levels of NDM metabolites were formed for both TOR and TAM, and *N*-demethylation of both compounds was primarily carried out by CYP3A4. We found that the formation of 4OH-NDM-TOR was catalyzed both by CYP2C9 and CYP2D6, whereas the formation of 4OH-TAM and endoxifen was specifically catalyzed by CYP2D6 in HLMs. Our results suggest that the potential contribution of CYP2D6 in the bioactivation pathway of TOR may be lower compared to TAM, and may have a different impact on clinical outcome than *CYP2D6* polymorphisms.

1. Introduction

Tamoxifen (TAM) is widely used as a selective estrogen receptor modulator (SERM) for estrogen receptor positive breast cancer [1]. Toremifene (TOR) has a structure that is similar to TAM, and has also been approved as a treatment for recurrent and advanced breast cancer and postoperative adjuvant endocrine therapy [2,3]. However, recent studies have suggested wide variability in the level of active metabolites of TAM in plasma that could affect both efficacy and toxicity of the drug. Such differences are in part based on genetic polymorphisms in the cytochrome P450 (CYP) metabolic system [4–8] as well as individual patient factors which have an influence on pharmacokinetic variations in general e.g., regulating function of drug absorption and disposition [9], and drug–drug interactions in use of concomitant drugs [10,11].

The main metabolic pathway of TAM has been investigated from a number of *in vitro* and *in vivo* studies (Fig. 1). Demethylation of the aminoethoxy side chain of TAM to *N*-desmethyl-TAM (NDM-TAM) appears to be the main route of TAM metabolism in humans [10]. 4-Hydroxy-TAM (4OH-TAM) is a relatively minor metabolite, but has been shown to have a high affinity for ERs and a 100-fold higher potency than TAM and NDM-TAM in suppressing estrogen-dependent cell proliferation [12,13]. 4-Hydroxy-*N*-desmethyl-TAM (endoxifen), a secondary metabolite of TAM, exhibits potency similar to 4OH-TAM with respect to ER binding affinity and suppression of estrogen-dependent cell growth [14]. Endoxifen is likely to bestow the greatest contribution to clinical efficacy due to a much higher (>6-fold) plasma concentration in breast cancer patients compared to 4OH-TAM [10].

From *in vitro* studies using human liver microsomes (HLMs), TAM is known to be metabolized by several CYP isoforms—NDM-TAM formation is mainly catalyzed by CYP3A4, and sequentially, endoxifen formation is mainly catalyzed by CYP2D6 [15–17]. Recently, it has been suggested that *CYP2D6* genotypes can

Abbreviations: TOR, toremifene; TAM, tamoxifen; SERM, selective estrogen receptor modulator; SSRI, selective serotonin reuptake inhibitor; 4OH, 4-hydroxy; NDM, *N*-desmethyl; 4OH-NDM, 4-hydroxy-*N*-desmethyl; CYP, cytochrome P450; HLM, human liver microsome; LC-MS/MS, liquid chromatograph-tandem mass spectrometry; SRM, selective reaction monitoring; CL_{int}, intrinsic clearance.

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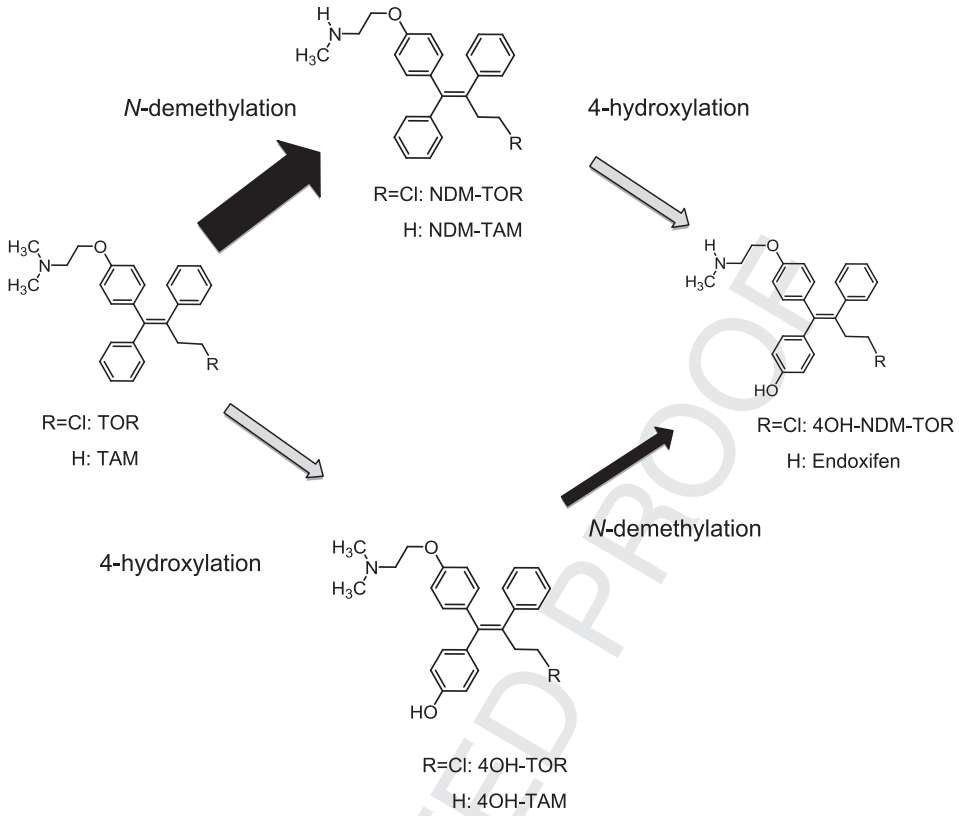

Fig. 1. Metabolic pathway of TOR and TAM in humans. Based on plasma concentration, the main metabolites of TOR and TAM are *N*-desmethyl-metabolites, while their 4-hydroxy-metabolites are at much lower levels in human plasma. The secondary metabolites of TOR and TAM, 4OH-NDM-TOR and endoxifen, are produced first by *N*-demethylation followed by 4-hydroxylation.

influence endoxifen steady-state plasma concentration, and thus the clinical benefits of TAM as well.

A number of clinical trials have reported the association between the *CYP2D6* genotype and clinical outcome of breast cancer patients on TAM therapy [4–7,9,18–20]. One of the first studies reported in 2005 demonstrated that homozygous carriers of *CYP2D6*4* (non-functional allele) had shorter relapse-free survival and disease-free survival compared with patients who were heterozygous or homozygous for the wild-type allele [4]. In Asians, it has been reported that *CYP2D6*10* (decreased functional allele) is significantly associated with worse clinical outcome in Japanese [9,20] and Chinese patients [7]. Additionally, use of selective serotonin reuptake inhibitors (SSRI), e.g., paroxetine, during TAM treatment reduces endoxifen levels [10] and is associated with an increased mortality risk from breast cancer [11].

Indeed, the relationship between *CYP2D6* polymorphisms and therapeutic effects of tamoxifen has been studied for a decade, but the results are actually contradictory and inconclusive because there is no conclusive evidence of relationship between endoxifen concentrations according to *CYP2D6* genotypes and clinical response.

Like TAM, TOR also undergoes biotransformation to NDM-, 4OH- and 4OH-NDM-metabolites *in vitro* (Fig. 1). However, it is unclear which enzymes are involved in catalyzing the conversion of TOR to these active metabolites. A previous study using human recombinant P450 microsomes showed the likely involvement of CYP3A4 and CYP2D6 in primary and secondary *N*-demethylation of TOR and 4OH-TOR, and that CYP2D6 and CYP2C19 may play an important role in the 4-hydroxylation metabolism of TOR and NDM-TOR [21]. However, the specific contributions of each of the other CYP isoforms to TOR metabolism remain unclear. Previous studies have shown that the main metabolite of TOR in human plasma is NDM-TOR, while levels of 4OH-TOR and 4OH-NDM-TOR are much lower or undetectable in plasma [21,22]. The latter two metabolites, however, have been reported to be detected in human urine samples at a higher dosing (a single 120 mg oral dose) [23], and it has been suggested that these potent metabolites are likely to contribute primarily or partially to antitumor effects due to their extremely high growth inhibition activities, particularly when high-dose TOR therapy is given.

In this study, we identified CYP isoforms associated with TOR metabolism in human recombinant P450 microsomes and HLMs using isozyme selective inhibitors. Additionally, we assessed the inhibitory effects of paroxetine on NDM-TOR 4-hydroxylation to confirm whether the CYP2D6 isoform contributes to metabolic activation. Indeed, intrinsic clearance of 4-hydroxylation of NDM-TOR was estimated in HLMs with intermediate or lower CYP2D6 activities. Given the important differences between TOR and TAM metabolic biotransformation in human cells *in vitro*, our studies should allow improved understanding of the mechanisms and factors that control TOR activation, and also support the expectation that drug–drug interactions or pharmacogenomics related to CYP2D6 are unlikely to influence TOR's clinical benefit.

2. Methods

2.1. Chemicals

Toremifene citrate, *N*-desmethyltoremifene citrate, 4-hydro xytoremifene, 4-hydroxy-*N*-desmethyltoremifene and internal standard (structural analog of TOR) were provided by Orion Corporation (Espoo, Finland). Tamoxifen citrate was purchased

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from Sigma–Aldrich (St. Louis, MO, US). *N*-Desmethyltamoxifen hydrochloride, 4-hydroxytamoxifen, 4-hydroxy-*N*-desmethyltamoxifen (1:1 E/Z mixture) were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada), and endoxifen was purified by HPLC from 4-hydroxy-*N*-desmethyltamoxifen (1:1 E/Z mixture).

For P450 inhibitors, furafylline, thio-TEPA, 8-methoxypsoralen, S-(+)-*N*-3-benzylnirvanol, quinidine anhydrous, diethyldithiocarbamate, and paroxetine hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, US). Ketoconazole was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Quercetin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Sulfaphenazole, (±)-bufuralol hydrochloride and (±)-1′-hydroxybufuralol maleate were purchased from Sumika Chemical Analysis Service, Ltd. (Tokyo, Japan). β-$NADP^+$, glucose-6-phosphate, and G6P dehydrogenase were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other reagents were of HPLC grade.

2.2. Growth inhibition of breast cancer cell lines

Estrogen receptor-expressing breast cancer cell lines MCF-7 and T-47D were used in the study. A total of 200 μL/well of MCF-7 (1.5×10^3 cells/mL) and T-47D (1.0×10^4 cells/mL) cell suspensions were seeded in RPMI1640 medium (Corning, NY, USA) containing 10% fetal bovine serum (Hana-Nesco Bio, Tokyo, Japan) and 50 μg/mL kanamycin (Life Technologies, Carlsbad, CA, USA) in 96-well plates. TOR, TAM and their metabolites were added in a concentration range of 10^{-11}–10^{-5} mol/L. After 7 days of cultivation in a CO_2 incubator, cells were stained with 0.05% methylene blue (Wako Pure Chemical Industries, Ltd.), and growth was evaluated by measuring absorbance at 660 nm with a Microplate reader (Bio-Rad Labs., Inc.).

2.3. Human liver microsomes and expressed P450s

HLMs were purchased from Biopredic International (Saint-Gregoire, France) and Celsis In Vitro Technologies (Baltimore, MD). Lymphoblastoid cells expressing human P450 isoforms (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4), and baculovirus insect cells expressing human P450 isoform (3A5) with reductase were purchased from Gentest, JapanBD (Japan). All microsomal samples were kept at −80 °C until use.

2.4. Incubation conditions

To determine conditions that were linear with regard to time of incubation and protein concentration, 1 or 5 μM of each substrate NDM-TOR and NDM-TAM was incubated in HLMs (0.1–1 mg protein/mL) with a NADPH-generating system at 37 °C across a range of incubation times (0–60 min). Each substrate (500 μmol/L) was prepared in methanol and serially diluted with DMSO or methanol to the required concentration. HLMs (0.1–1 mg protein/mL), reconstituted in a phosphate reaction buffer (pH, 7.4) and a NADPH generating system (2 mM NADP, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$, and 2 U/mL glucose 6-phosphate dehydrogenase), were pre-incubated at 37 °C for 10 min before the reaction was initiated by the addition of duplicate mixtures of 10 or 50 μM of NDM-TOR or NDM-TAM substrate (final substrate concentration; 1 or 5 μM; total reaction volume of 100 μL). After the required incubation period, the reaction was terminated by the addition of cold acetonitrile containing an internal standard (100 μL of 2 nmol/L TOR-IS). Samples were centrifuged at 2000 rpm for 10 min filtered through a 0.45 μm Multiscreen membrane filter (Merck Millipore, MA, US) and diluted with an equal volume of water. An aliquot of diluent was injected into the liquid chromatograph-tandem mass spectrometry (LC-MS/MS) system.

Based on the results obtained, a 30 min incubation time and a final microsomal protein concentration of 0.1 mg/mL represented the conditions that were used in subsequent experiments.

2.5. LC-MS/MS analysis

The LC-MS/MS method was performed with the Prominance HPLC system (Shimadzu, Japan) and electrospray ionization-triple quadruple tandem mass spectrometer TSQ Vantage (Thermo fisher scientific, US). Xcalibur (ver. 2.0.7 sp1, Thermo fisher scientific, US) was used to control the HPLC and mass spectrometer and to process the data. The chromatographic separation was performed on a Capcell Pak C_{18} AQ type (2.0 mm × 75 mm, particle size 3 μm, Shiseido, Kyoto, Japan) at a column temperature of 40 °C. The mobile phase consisted of solvent A (10 mM formate ammonium, pH 4.0) and solvent B (methanol). A flow rate of 200 μL/min was used for sample analysis. The gradient elution was as follows: 55% solvent B for the initial condition, followed by a gradual increase in the solvent B percentage to 60% at 3.2 min, 85% at 5.0 min and 90% at 5.4 min and maintaining for 3.6 min, then re-equilibrating to the initial conditions over 0.1 min and maintaining for 4 min. The temperature of the autosampler was kept at 4 °C. Detection was performed with MS/MS in the positive ion mode using selective-reaction monitoring (SRM). Nitrogen was used as a nebulizer and a drying gas, and argon was used as a collision gas. The tune setting was as follows: spray voltage of 3000 V, vaporizer temperature of 450 °C, capillary temperature of 300 °C, sheath gas pressure of 50 psi, aux gas of 15 psi, and a Q2 collision gas pressure of 1.2 mTorr. The precursor-product ion transitions for SRM and MS/MS parameter settings are shown in the Supplemental table. Quantitation ranges were set to 0.5–1000 nmol/L for TAM, TOR and their NDM-metabolites; and 0.05–100 nmol/L for 4OH-and 4OH-NDM-metabolites.

2.6. Expressed human P450s

To further probe the specific isoforms involved in TOR and TAM primary and secondary metabolism, 1 μM of each substrate (TOR, TAM, NDM-TOR, NDM-TAM, 4OH-TOR and 4OH-TAM) was incubated with microsomes from expressed human CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and CYP3A5 (0.05 mg protein/mL, 28–220 pmol P450/mg protein in phosphate reaction buffer, pH 7.4) at 37 °C for 30 min (final incubation volume 100 μL). All other conditions were the same as those described for HLMs. To estimate the contribution of each P450 isoform to the metabolism of TOR and TAM, percentage total normalized rates (%TNR) were calculated using the method described by Rodrigues (1999) [24]. For each recombinant P450s, the reaction rate was normalized by multiplying the rate with the specific content of the corresponding P450 (pmol of P450/mg of microsomal protein) in human liver microsomes, and was expressed as a normalized rate (NR; pmol/min/mg microsomal protein). The NR values for each P450 were summed to obtain the total normalized rate (TNR). Finally, the NR for each P450 was expressed as a percentage of the TNR:

$$\%\mathrm{TNR} = \frac{\mathrm{NR}}{\mathrm{TNR}} \times 100 = \frac{\mathrm{pmol/min/pmol\ rP450n \cdot pmol\ mP450n/mg}}{\sum(\mathrm{pmol/min/pmol\ rP450n \cdot pmol\ mP450n/mg})} \times 100$$

rP450n; each recombinant P450, and *mP450n*; the specific content of each P450 in human liver microsomes.

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In this study, we adapted the data of immunologically determined P450 isoform liver contents as described by Rodrigues (1999); i.e., 8% for CYP1A2, 13% for CYP2A6, 7% for CYP2B6, 12% for CYP2C8, 18% for CYP2C9, 4% for CYP2C19, 2% for CYP2D6, 9% for CYP1E1, 20% for CYP3A4 and 0.2% for CYP3A5 [24], except for CYP1A1 expressed mainly in the extrahepatic tissues [25].

2.7. Inhibition experiments

Formation rates of metabolites from TAM, TOR and their NDM- and 4OH-metabolites (1 μM) were evaluated in the absence (control) and presence of known P450 isoform-specific inhibitors. The inhibitors used were 5 μM furafylline (CYP1A2), 50 μM thio-TEPA (CYP2B6), 5 μM 8-methoxypsoralen (CYP2A6), 5 μM quercetin (CYP2C8), 5 μM sulfaphenazole (CYP2C9), 1 μM S-(+)-*N*-3-benzylnirvanol (CYP2C19), 1 μM quinidine (CYP2D6), 50 μM diethyldithiocarbamate(CYP2E1), and 0.1 μM ketoconazole (CYP3A4/5). All inhibitors and controls were first pre-incubated with a NADPH-generating system and HLMs (0.1 mg protein/mL) at 37 °C for 10 min before the reaction was initiated by addition of each substrate at 37 °C and incubation for 30 min. Formation rates of metabolites from NDM-TOR and NDM-TAM were evaluated in the presence of paroxetine (1 μM), an SSRI, and quinidine (1 μM), a typical CYP2D6 inhibitor. Incubation conditions were the same as described above.

2.8. Assay of CYP2D6 activity in HLMs

CYP2D6 activity in HLMs was confirmed by measuring the 1′-hydroxylation velocity (pmol/min/mg protein) of bufuralol, one of the probe substrates for CYP2D6. Substrate at a range of concentrations (0–60 μM) was incubated for 20 min at 37 °C with 0.1 mg protein/mL and a NADPH-generating system. Methods for further processing of the samples and the HPLC assay were the same as described above except for the chromatogram gradient and MS/MS tune methods. Conditions for the gradient elution were as follows: 10% solvent B for the initial condition, followed by a gradual increase in the solvent B percentage to 20% at 1.5 min, 75% at 4 min and 90% at 4.5 min and maintaining for 1.5 min, then re-equilibrating to the initial conditions over 0.1 min and maintaining for 2 min. Total flow was 300 μL/min. The MS/MS tune setting was as follows: spray voltage of 3000 V, vaporizer temperature of 300 °C, capillary temperature of 300 °C, sheath gas pressure of 30 psi, aux gas of 35 psi, and a Q2 collision gas pressure of 1.5 mTorr. The precursor-product ion SRM transition and MS/MS parameter settings were m/z 635.7 → m/z 607.5 for 1′-hydroxybufuralol.

2.9. Kinetic analyses in HLMs

Kinetic analyses of the metabolism of NDM-TOR and NDM-TAM and their metabolites, 4OH-NDM-TOR and 4OH-NDM-TAM, were determined in characterized HLMs (two individuals and one 20-donor pool). Substrate at a range of concentrations (0–80 μM) was incubated for 30 min at 37 °C with protein at a concentration of 0.1 mg/mL and a NADPH-generating system. Methods for further processing of the samples and the HPLC assay were the same as described above.

2.10. Data analysis

Apparent kinetic constants were estimated by nonlinear regression analysis using Phoenix WinNonlin Software Version 6.3 (Certara, St. Louis, MO). Formation rates (V) of metabolites versus substrate concentrations (C) were fit to the Michaelis–Menten kinetics $V = V_{max} \times C/(K_m + C)$ equation. K_m and V_{max} were estimated as mean ± S.D. (n = 3 experiments). *In vitro* intrinsic clearance (CL_{int}) represented as V_{max}/K_m. CL_{int} in HLMs with intermediate and lower CYP2D6 activity was compared using an unpaired two-tailed Student's t test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Growth inhibition activities of toremifene, tamoxifen and their metabolites

We conducted *in vitro* assays to determine and compare the relative potency of TOR, TAM and their NDM- and 4-OH- metabolites on growth inhibition of ER-positive breast cancer cells after 7 days of exposure (Table 1). The IC_{50} values of TOR and TAM in MCF-7 were 225 nM and 81.8 nM, respectively. Cell growth inhibition activities of NDM-TOR and NDM-TAM were at levels similar to each of their parent compounds; IC_{50} values in MCF-7 cells were 347 nM and 96.2 nM, respectively. On the other hand, 4OH- and 4OH-NDM metabolites of TOR and TAM showed extremely high potency towards cell growth inhibition compared to their respective parent compounds and to NDM-metabolites, with IC_{50} values that were approximately 100-fold higher than TOR and NDM-TOR (2.20 and 2.30 nM in MCF-7 cells, respectively). The variation in inhibitory activity between the metabolites showed a similar pattern in T-47D cells. Although TOR and its metabolites have slightly weaker potency than TAM and its metabolites *in vitro*, the relative activity of 4OH- and 4OH-NDM-metabolites of TOR and TAM were drastically higher than their respective parent compounds.

3.2. Metabolism of toremifene and tamoxifen to their primary metabolites

The metabolism of TOR and TAM to NDM-and 4OH- metabolites was examined using human recombinant P450 isoforms and CYP isoform-selective inhibitors in HLMs (Fig. 2). *N*-Demethylation velocity (pmol/min/pmol P450) of TOR or TAM was higher for recombinant CYP1A1, 2D6, 3A4, and 3A5. To estimate the relative contributions of each P450 isoforms to the TOR and TAM metabolic pathway, %TNR was calculated. CYP3A4 contributed 88% to the formation of NDM-TOR, whereas CYP3A4 and CYP2D6 contributed 66% and 25%, respectively, to the formation of NDM-TAM. From the inhibition assay, *N*-demethylation velocity for TOR or TAM decreased to 20% of control with KCZ (CYP3A4/5), with smaller inhibition seen using DDC (CYP2E1), thio-TEPA (CYP2B6) and QUER (CYP2C8). Thus, CYP3A4 has a greater contribution to the formation of NDM-metabolites.

Table 1
Inhibition of breast cancer cell growth of TOR and TAM and their metabolites.

Compound	IC_{50} (nM)		Relative activity		
	MCF-7	T-47D	MCF-7	T-47D	Mean
TOR	225	500	1	1	1
NDM-TOR	347	569	0.648	0.879	0.764
4OH-TOR	2.20	5.32	102	94.0	98.0
4OH-NDM-TOR	2.30	5.93	97.8	84.3	91.1
TAM	81.8	90.2	1	1	1
NMD-TAM	96.2	196	0.850	0.460	0.655
4OH-TAM	0.510	1.25	160	72.2	116
Endoxifen	1.45	2.49	56.4	36.2	46.3

ER positive breast cancer cell lines MCF-7 and T-47D were exposed to TOR and TAM and their primary and secondary metabolites for 7-days. Relative activity was estimated by dividing the IC_{50} of the parent compounds by that for each of their metabolites.

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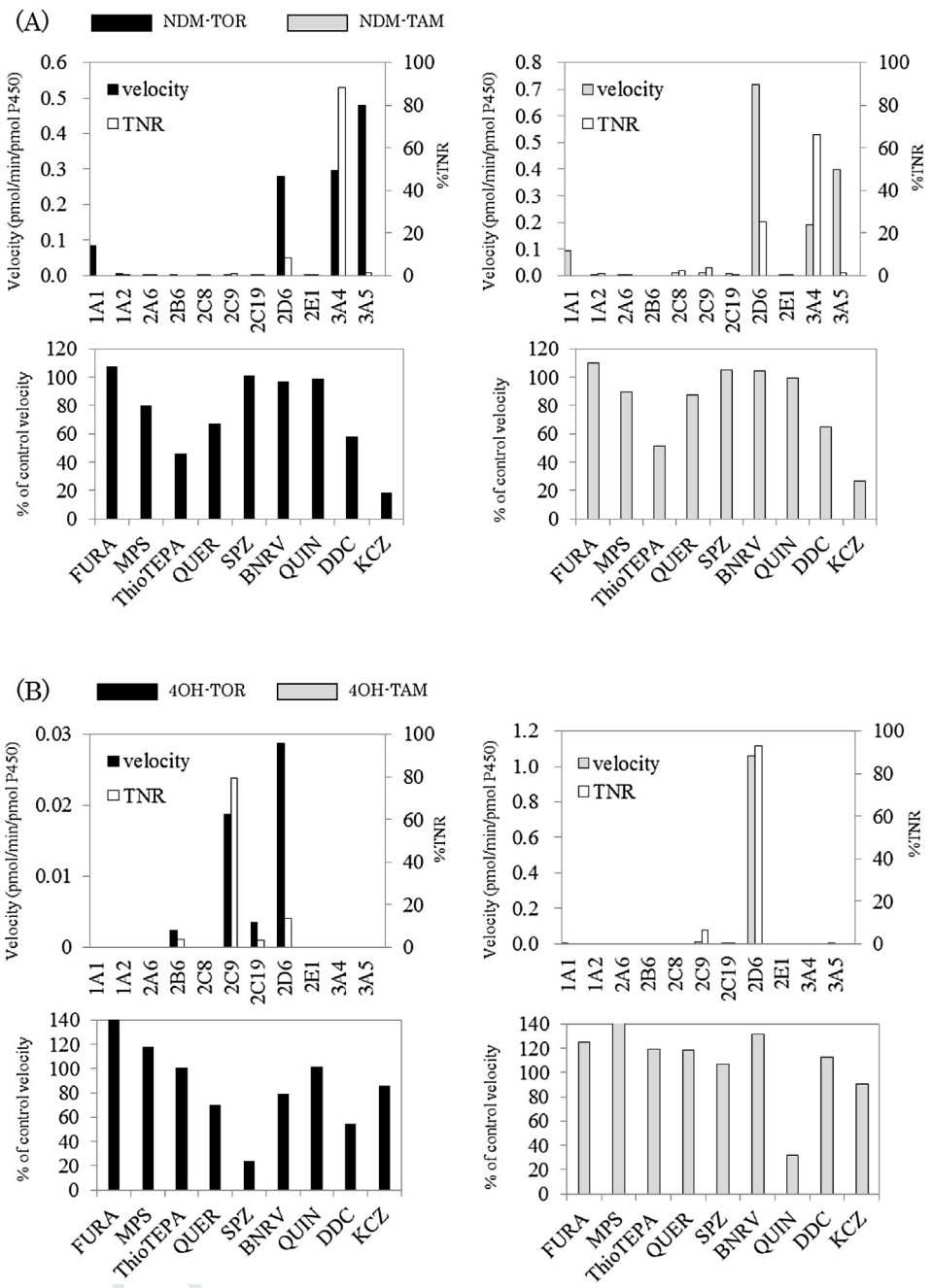

Fig. 2. Primary metabolism of TOR and TAM by 11 recombinant CYP isozymes (upper panel) and inhibition by CYP isoform-selective inhibitors (lower panel). The formation rate of *N*-demethyl metabolites (A) and 4-hydroxylation metabolites (B) were assessed with the LC-MS/MS system. In recombinant CYP assays, the left *Y*-axis is the velocity (pmol/min/pmol P450) normalized with the P450 content in each recombinant CYP (closed bar). The right *Y*-axis is the %TNR that means the metabolic contribution of each isoform normalized with the relative content of P450 in pooled HLMs (open bar). In inhibition assays, the percent of formation rate (pmol/min/mg protein) to the control sample (with solvent of inhibitors) were shown. Respective inhibitors (abbreviations, CYP isoforms, final concentration) were: Furafylline (FURA, CYP1A2, 5 μM), 8-Methoxypsoralen (MPS, CYP2A6, 50 μM), Thio-TEPA (Thio-TEPA, CYP2B6, 5 μM), Quercetin (QUER, CYP2C8, 5 μM), Sulfaphenazole (SPZ, CYP2C9, 5 μM), S-(+)-*N*-3-Benzylnirvanol (BNRV, CYP2C19, 1 μM), Quinidine (QUIN, CYP2D6, 1 μM), Diethyldithiocarbamate (DDC, CYP2E1, 50 μM) and Ketoconazole (KCZ, CYP3A4/5, 0.1 μM).

4-Hydroxylation velocity of TOR was higher for recombinant CYP2B6, 2C9, 2C19 and 2D6, whereas 4OH-TAM was catalyzed selectively by CYP2D6. From the %TNR estimation, CYP2C9 and CYP2D6 contributed 79% and 13%, respectively, to the formation of 4OH-TOR. On the other hand, CYP2D6 largely contributed 93% to the formation of 4OH-TAM. From the inhibition assay, the formation velocity of 4OH-TOR decreased to 24% of control with SPZ (CYP2C9), with less inhibition seen with DDC (CYP2E1) and QUER (CYP2C8), whereas 4OH-TAM metabolism was inhibited only by QUIN (CYP2D6). These results show that 4-hydroxylation of TOR is catalyzed mainly by CYP2C9 and not CYP2D6, which showed a greater contribution to 4-hydroxylation of TAM.

3.3. Metabolism of toremifene and tamoxifen to their secondary metabolites

The conversion of NDM- or 4OH- primary metabolites to 4OH-NDM-metabolites was examined using human recombinant P450

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isoforms and CYP isoform-selective inhibitors in HLMs (Fig. 3). *N*-Demethylation velocity of 4OH-TOR and 4OH-TAM were higher for recombinant CYP1A1, 2D6, 3A4, and 3A5. From the %TNR estimation, CYP3A4 largely contributed 90% to *N*-demethylation of 4OH-TOR. For *N*-demethylation of 4OH-TOR, CYP3A4 contributed 69% to, in addition CYP2D6 and 2C9, combined, contributed 24%. From the inhibition assay, the velocity decreased to 20% of control with KCZ (CYP3A4/5), with smaller inhibition noted with DDC (CYP2E1), thio-TEPA (CYP2B6) and QUER (CYP2C8). Thus, CYP3A4 contributes more to the formation of NDM-metabolites from 4OH-metabolites in the same manner as *N*-demethylation of TOR and TAM.

4-Hydroxylation velocity of NDM-TOR was higher for recombinant CYP2D6 and CYP2C9, whereas 4OH-TAM was catalyzed only by CYP2D6. From the %TNR estimation, CYP2D6 and CYP2C9 contributed 79% and 21%, respectively, to the 4-hydroxylation of NDM-TOR. On the other hand, CYP2D6 significantly contributed 99% to the 4-hydroxylation of NDM-TAM. From the inhibition assay, the formation velocity of 4OH-NDM-TOR decreased to 60% of control with either SPZ (CYP2C9) or QUIN (CYP2D6), whereas

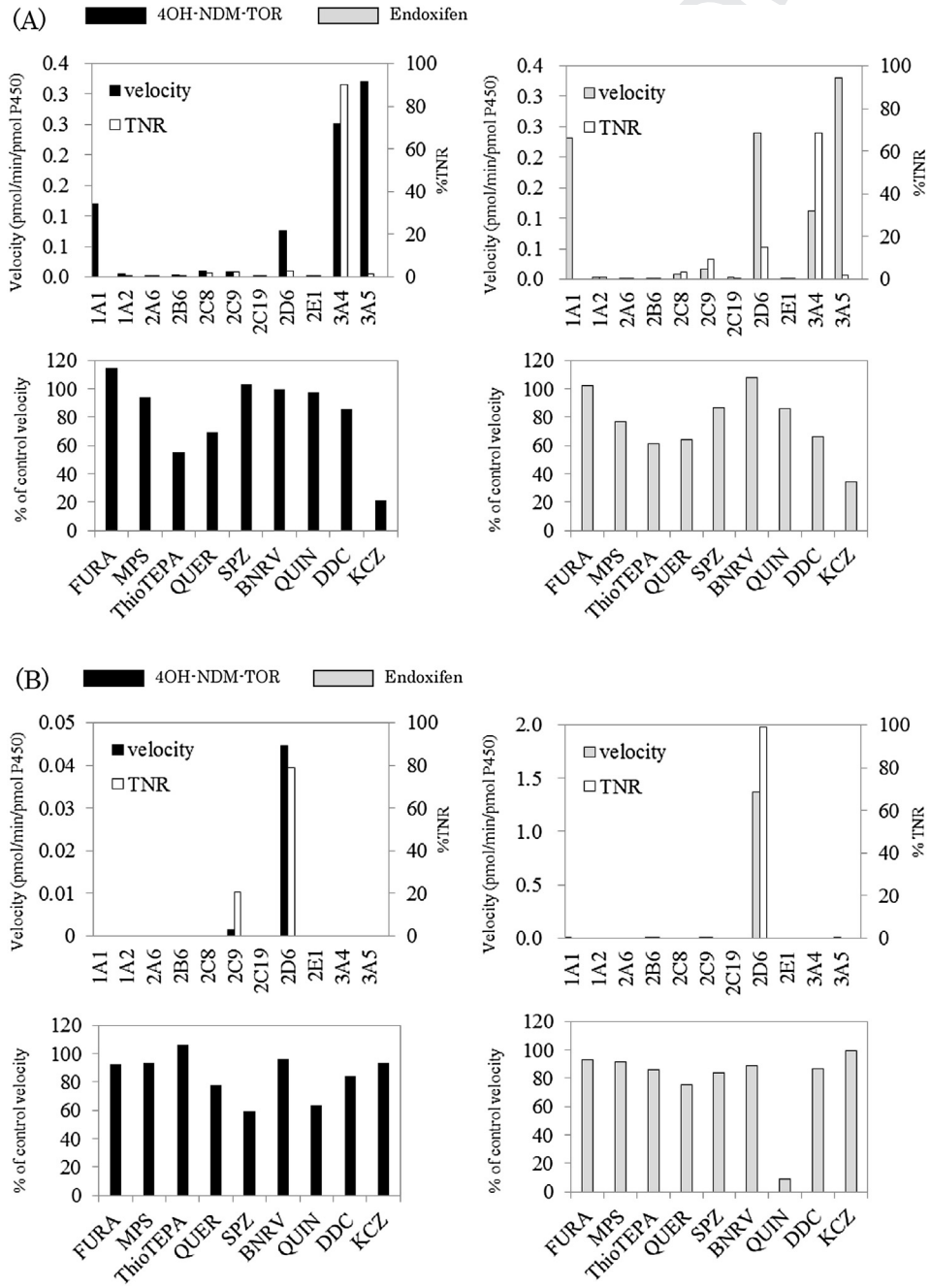

Fig. 3. Secondary metabolism of TOR and TAM by 11 recombinant CYP isozymes (upper panel) and inhibition by CYP isoform-selective inhibitors (lower panel). The 4OH-NDM-metabolite formation rates for *N*-demethylation from 4OH-metabolites (A) and 4-hydroxylation from NDM-metabolites (B) were determined by the LC-MS/MS system. In recombinant CYP assays, the left *Y*-axis is the velocity (pmol/min/pmol P450) normalized with the P450 content in each recombinant CYP (closed bar). The right *Y*-axis is the %TNR that means the metabolic contribution of each isoform normalized with the relative content of P450 in pooled HLMs (open bar). In inhibition assays, the percent of formation rate (pmol/min/mg protein) to the control sample (with solvent of inhibitors) were shown. Respective inhibitors (abbreviations, CYP isoforms, final concentration) were the same as those in Fig. 2.

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Table 2
CYP isoforms involved in the metabolism of TOR and TAM determined in rCYPs assay and inhibition assay.

Primary metabolite	*N*-demethylation		4-Hydroxylation	
	NDM-TOR	NDM-TAM	4OH-TOR	4OH-TAM
Recombinant CYPs[a]	3A4	3A4, 2D6	2C9, 2D6	2D6
Inhibition assay	3A4/5, 2B6, 2E1, 2C8	3A4/5, 2B6, 2E1	2C9, 2E1, 2C8	2D6
Higher contribution	3A4	3A4	2C9	2D6
Secondary metabolite	4OH-NDM-TOR	Endoxifen	4OH-NDM-TOR	Endoxifen
Recombinant CYPs[a]	3A4	3A4, 2D6	2D6, 2C9	2D6
Inhibition assay	3A4/5, 2B6, 2C8,	3A4/5, 2B6, 2C8, 2E1	2D6, 2C9	2D6
Higher contribution	3A4	3A4	2D6, 2C9	2D6

[a] Recombinant CYPs was determined from the formation velocity normalized by relative contents of each P450 in HLM as %TNR.

endoxifen production was strongly inhibited by QUIN (CYP2D6) to less than 10% of control. These results show that CYP2D6 contributes highly specifically to endoxifen production from NDM-TAM, while in contrast, 4-hydroxylation of NDM-TOR was catalyzed by both CYP2C9 and CYP2D6.

These primary and secondary metabolic assay results are summarized in Table 2. We found that *N*-demethylation of TOR and TAM were catalyzed equally by CYP3A4, whereas 4-hydroxylation of TOR was mainly catalyzed by CYP2C9 and not CYP2D6. Interestingly, 4-hydroxylation of NDM-TOR was catalyzed by both CYP2C9 and CYP2D6, while CYP2D6 contributed strongly to endoxifen production.

3.4. Contribution of CYP2D6 to metabolic activation of toremifene and tamoxifen

To evaluate the inhibitory effects of SSRIs, 4OH-NDM-metabolite formation in the presence of paroxetine was examined using HLMs (Fig. 4). The production rate of 4OH-NDM-TOR was partially inhibited by 27% with 1 μM paroxetine and 30% with 1 μM quinidine, a typical inhibitor of CYP2D6. Endoxifen production was greatly influenced by CYP2D6 inhibitors, with significantly 84% inhibition seen with 1 μM paroxetine and 92% inhibition with 1 μM quinidine.

To determine if production of 4OH-NDM-TOR and endoxifen were influenced by individual differences in CYP2D6 activity, intrinsic clearance (CL_{int}) was estimated by kinetics parameters (V_{max} and K_m) for the 4-hydroxylation of NDM-TOR and NDM-TAM, and the CLint between two individual HLMs (Lot No. FND and QJH), was compared (Table 3). CYP2D6 activities of these HMLs were assessed by CL_{int} for bufuralol 1′-hydroxylation. FND showed intermediate CYP2D6 activity as same as 20-donor pooled HML, whereas QJH showed lower activity, as the CL_{int} values (L/min/μg protein) were 25.6 (Lot No. FND), 2.67 (Lot No. QJH) and 25.2 (20-donor pool), respectively.

Regarding the formation of 4OH-NDM-TOR, estimates of the kinetics parameters were V_{max}: 3.77 ± 1.43 pmol/min/mg protein and K_m: 17.1 ± 5.9 μM in FND, whereas for QJH, they were V_{max}: 0.760 ± 0.180 pmol/min/mg protein and K_m: 2.35 ± 0.96 μM. Regarding the formation of endoxifen, estimates of the kinetics parameters were V_{max}: 31.8 ± 4.0 pmol/min/mg protein and K_m: 11.6 ± 1.6 μM in FND, whereas for QJH, they were V_{max}: 4.11 ± 0.49 pmol/min/mg protein and K_m: 4.15 ± 0.72 μM. CL_{int} (V_{max}/K_m) for NDM-TOR 4-hydroxylation was 0.221 ± 0.028 L/min/μg protein in FND and 0.355 ± 0.126 L/min/μg protein in QJH. The value of CL_{int} for 4-hydroxylation of NDM-TAM in QJH was 1.01 ± 0.21 L/min/μg protein, which was 2.7-fold lower than in FND (2.77 ± 0.56 L/min/μg protein). CL_{int}s in HLMs with low and intermediate CYP2D6 activity resulted in a significant difference in endoxifen formation, but not in 4OH-NDM-TOR formation.

4. Discussion

In the current study, we identified CYPs involving TOR metabolic activation using recombinant P450s and selective inhibitors. Additional findings provided from *in vitro* kinetics studies showed that CYP2D6 status could not contribute to TOR activation, whereas it could play important roles in TAM activation.

With regard to the expressed CYPs and the inhibition assay study, the NDM metabolite was found to be the most abundant for both TOR and TAM. This metabolite was formed by CYP3A4 at a higher rate than the 4OH metabolite (Fig. 2). The 4OH metabolites were the most effective, however, showing 100-fold higher growth inhibition in MCF-7 and T-47D cancer cells than their parent compounds and NDM metabolites (Table 1). 4OH-TOR was produced from TOR mainly by CYP2C9, but not CYP2D6 as with TAM.

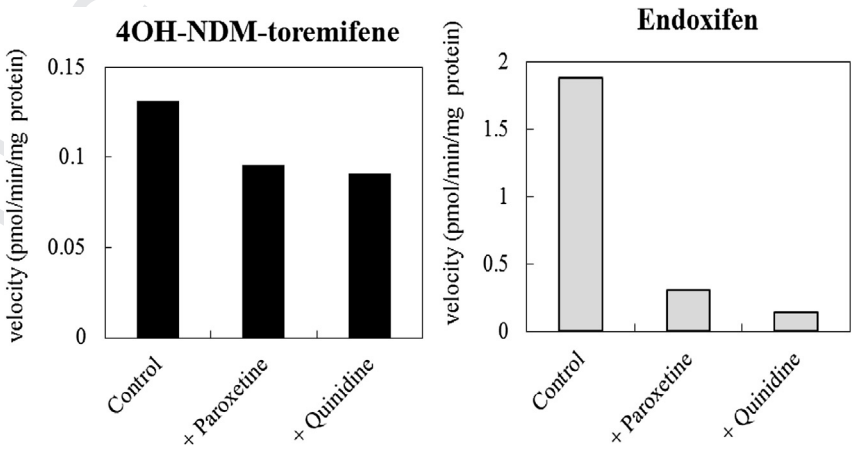

Fig. 4. Inhibitory effects of 4-hydroxlation of NDM-TOR and NDM-TAM by paroxetine in HLMs. NDM-TOR (2.5 μM) and NDM-TAM (2.5 μM) were incubated with paroxetine (1 μM) or quinidine (1 μM) in HLMs (0.1 mg protein/mL) for 30 min, and their 4-hydroxy metabolites, 4OH-TOR and endoxifen, were detected with LC-MS/MS.

Table 3
Pharmacokinetic parameters for 4-hydroxylations of NDM-TOR and NDM-TAM in two HLMs with different CYP2D6 activities.

4-Hydroxylated Metabolite	HML lot	CYP2D6 activity	K_m (μM)	V_{max} (pmol/min/mg protein)	CL_{int} (L/min/μg protein)
4OH-NDM-TOR	FND	Intermediate	17.1 ± 5.9	3.77 ± 1.43	0.221 ± 0.028
	QJH	Low	2.35 ± 0.96	0.760 ± 0.180	0.355 ± 0.126
Endoxifen	FND	Intermediate	11.6 ± 1.6	31.8 ± 4.0	2.77 ± 0.56
	QJH	Low	4.15 ± 0.72	4.11 ± 0.49	1.01 ± 0.21*

Values are mean ± S.D. ($n = 3$). *$P < 0.05$ (vs. FND).
CYP2D6 activity (intermediate or low) in HLMs was compared with CL_{int} values for bufuralol 1′-hydroxylation in HLMs from a 20-donor pool, as the actual CL_{int} values (L/min/μg protein) were 25.6 (Lot No. FND), 2.67 (Lot No. QJH) and 25.2 (20-donor pool), respectively.

4OH-NDM-TOR showing strong activity as well 4OH-TOR was converted from NDM-TOR by CYP2D6 and CYP2C9, whereas endoxifen formation was specifically catalyzed by CYP2D6 (Figs. 2–4). These results suggest a lower contribution of CYP2D6 in 4-hydroxylations of TOR and NDM-TOR (4OH- and NDM-4OH-metabolites) than that in the formation of 4OH-TAM and endoxifen.

Plasma concentrations of these active TOR metabolites have not yet been determined. For both TAM and TOR, the main metabolite in the plasma of patients is the NDM metabolite [10,21,22], which is also supported by our *in vitro* study. Combining the plasma concentration and the anti-estrogenic activity of these metabolites, endoxifen is thought to be the most important with regard to eliciting the clinical effects of TAM therapy *in vivo*.

In recent clinical studies, the plasma concentrations of endoxifen and 4OH-TAM have revealed wide inter-individual variation among patients carrying *CYP2D6* genetic variants [5,6,8,9,26,27] Allelic variants *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5* and *CYP2D6*6* are well known as non-functional alleles encoding proteins with little enzymatic activity. Those who are homozygous for these null alleles represent about 5–10% of the Caucasian population, and less than 1% of Asians [28,29]. The steady-state plasma concentrations of endoxifen are four-fold lower in patients with a homozygous genotype, and two-fold lower in those with heterozygous genotype than in those with a wild-type genotype [5,26,27]. On the other hand, allelic variants *CYP2D6*9* and *CYP2D6*10*, which are decreased-functional alleles, are frequently found in the Asian population (allele frequency of 40%) [30]. Plasma levels of endoxifen are three-fold lower in patients with a homozygous genotype than in those with a wild-type genotype [6,8,9].

We evaluated 4-hydroxylation from NDM-TOR and NDM-TAM in microsomes from donors of different CYP2D6 status, from which predictions were made about clinical levels of 4OH-NDM-TOR and endoxifen on intrinsic clearance values *in vitro* comparing low and intermediate CYP2D6 activities (Table 3). Kinetics data revealed a 2.7-fold difference in clearance values between the two CYP2D6 activities, and these results were consistent with clinical plasma levels of endoxifen between people with wild type *CYP2D6* and those with the genetic variant. In contrast, no difference was found in the formation of 4OH-NDM-TOR due to the lower contribution of CYP2D6 to metabolic activation. Interestingly, our kinetics data showed a difference in K_m value for NDM-TOR 4-hydroxylation in two lots of human liver microsomes, QJH and FND, which have different CYP2D6 activity. From the recombinant P450 and inhibition assay, CYP2C9 should also contribute to 4-hydroxylation of NDM-TOR (Fig. 3). Furthermore, another kinetics study in 4-hydroxylation of NDM-TOR using recombinant human CYP2D6 and CYP2C9 provided K_m values 18.3 μM and 4.3 μM, respectively (data not shown). These results are consistent with the lower K_m in QJH, which have poorer CYP2D6 activity, and no difference in clearance for NDM-TOR 4-hydroxylation between those two micrsomes. CYP2C9 is also known to be a polymorphic, allelic variant of *CYP2C9*2* and *CYP2C9*3*, which are present in 30% of Caucasians, but much less common in Asian populations [31]. People with PM type variants of both *CYP2D6* and *CYP2C9* comprised less than 1% of the Greek population [32]. Although rare, these decreased-functional alleles, both for *CYP2D6* and *CYP2C9*, might influence TOR pharmacokinetics in people carrying them.

However, 4OH-NDM-TOR has been detected in human urine [23], although plasma steady-state concentration and its clinical contribution to TOR therapy are still unknown. A previous study evaluated plasma concentration of TOR and its metabolites in healthy volunteers administered 80 mg every 15 days, finding that TOR and NDM-TOR were at extremely high levels (more than 2000 and 6000 nM, respectively), and 4OH-TOR was at a relatively lower level (about 20 nM), whereas 4OH-NDM-TOR was not detected at all (less than 4 nM) [21]. It is believed that TOR itself and NDM-TOR are at much higher concentrations than TAM due to the dosing amount of 40–120 mg/day, and thus the former are likely sufficient in eliciting anticancer effects *in vivo*.

In conclusion, the potential contribution of CYP2D6 to the bioactivation pathway of TOR appears to be lower than for TAM, and may impact therapeutic effects differently due to *CYP2D6* polymorphisms or drug–drug interactions. Future investigations are needed to elucidate the associations between TOR pharmacokinetics and *CYP2D6* polymorphism and to evaluate clinical outcomes.

Authorship contributions

Participated in research design: Miyuki Watanabe, Noriko Watanabe, Sakiko Maruyama and Takashi Kawashiro.

Conducted experiments: Miyuki Watanabe and Sakiko Maruyama.

Performed data analysis: Miyuki Watanabe and Sakiko Maruyama.

Wrote or contributed to the writing of the manuscript: Miyuki Watanabe, Noriko Watanabe, Sakiko Maruyama and Takashi Kawashiro.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dmpk.2015.05.004.

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