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INVOLVEMENT OF CYTOCHROME P450 3A ENZYME FAMILY IN THE MAJOR METABOLIC PATHWAYS OF TOREMIFENE IN HUMAN LIVER MICROSOMES

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Abstract—The anti-estrogen toremifen-Fc-1157a or 4-chloro-1,2-diphenyl-1-[4-[2(*N*,*N*-dimethylamino)ethoxy]-phenyl]-1-butene is now used for the treatment of breast cancer. This drug is extensively metabolized by cytochrome P450 dependent hepatic mixed function oxidase in man, yielding mainly the *N*-demethyl-(DMTOR), 4-hydroxy-(4OH-TOR) and deamino-hydroxy- (TOR III) toremifene metabolites. The specific forms of cytochrome P450 involved in these oxidation reactions were examined in 32 human liver microsomal preparations previously characterized with respect to their contents of several known P450 enzymes. Toremifene was demethylated with an apparent K_m of 124 μM while it was hydroxylated with an apparent K_m of 139 μM. The metabolic rates were 71 ± 56, 13 ± 9 and 15 ± 4 pmol/min/mg microsomal protein, respectively, for DMTOR, 4-OH-TOR and TOR III. The N-demethylation activity was strongly correlated with estradiol 2-hydroxylation ($r = 0.75$), nifedipine oxidation ($r = 0.86$), tamoxifen N-demethylation ($r = 0.73$), testosterone 6β-hydroxylation ($r = 0.78$) and erythromycin N-demethylation ($r = 0.84$), all these monooxygenase activities known to be supported by CYP3A4 isoform. Furthermore, the CYP3A content of liver microsomal samples, measured by western blot analysis using a monoclonal anti-human CYP3A4 antibody, was strongly correlated with DMTOR formation ($r = 0.80$). Compounds such as cyclosporin, triacetyl-oleandomycin and testosterone inhibited the N-demethylation of toremifene metabolism at 80, 89 and 56% vs control, respectively, while the formation of TOR III was inhibited at 78, 82 and 73% vs control and the 4-hydroxylation pathway was inhibited no more than about 50% vs control. Prior incubation of microsomes with 100 μM gestodene, known to be a selective mechanism-based inhibitor of CYP3A4 in the presence of NADPH, led to 76 ± 6 and 76 ± 5% (N = 5 samples) reductions in the N-demethylation and formation of TOR III, respectively. Polyclonal antibody directed against human CYP3A enzymes inhibited formation of DMTOR and TOR III by 60 and 46%, respectively. The metabolism of toremifene was not activated by α-naphthoflavone. Finally, the use of yeasts genetically engineered for expression of human P4501A1, 1A2, 2C9 and 3A4 allowed us to demonstrate that DMTOR and TOR III formations are mediated by P4501A and 3A4 enzymes and by contrast these enzymes are not involved in the 4-hydroxylation pathway. All these results taken together suggest that the major metabolic pathways of toremifene, namely N-demethylation and TOR III formation, are mediated mainly by cytochrome CYP3A4 enzyme in the human liver microsomes owing to the relative hepatic contents in P4501A and 3A.

Key words: toremifene metabolism; anticancer drug; human liver microsomes; N-demethylation; cytochrome CYP3A4

TOR‖ is a new triphenylethylene anticancer drug developed by the Orion-Farmos group. As might be expected from its chemical structure (Table 1), (4-chloro-1,2-diphenyl-1-[4-[2(*N*,*N*-dimethylamino)-ethoxy]-phenyl]-1-butene), the pharmacological properties are very similar to these reported for TAM [1–3], a drug of the same anticancer class as TOR. This new anticancer drug has been developed to offer advantages of selectivity and safety over TAM which has for years been the only widely used anti-estrogen for hormonal control of endocrine-related breast tumors [4]. Although TOR and TAM have very similar *in vitro* and *in vivo* binding properties for the cytosolic estrogen receptor, TOR was demonstrated to be able to reverse multidrug resistance [5] acquired against TAM. The elimination half-life was about 5–6 days for the two triphenylethylene derivatives [2]. TOR is metabolized in the rat [6] and in humans [1], mainly by

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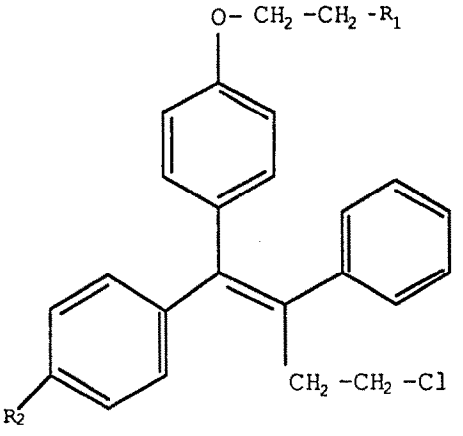
‖ Abbreviations: TOR, toremifene; DMTOR, *N*-demethyl-toremifene; 4-OH-TOR, 4-hydroxy-toremifene; TOR III, deamino-hydroxy toremifene; TST, testosterone; NIF, nifedipine; CsA, cyclosporin A; TAO, triacetyl-oleandomycin (troleandomycin); DDYA, 11-dodecynoic acid; α-NF, α-naphthoflavone (7,8-benzoflavone); TAM, tamoxifen; E2, 17-β-estradiol; 17α-E-E2; 17α-ethynyl-estradiol; PBS: potassium phosphate-buffered saline.

Table 1. Chemical structure of TOR and its main metabolites



	Abbreviations	R_1	R_2
Toremifene	TOR	N-$[CH_3]_2$	H
N-Demethyl-toremifene	DMTOR (TOR I)	NH-CH_3	H
4-OH-Toremifene (TOR II)	4-OH-TOR	N-$[CH_3]_2$	OH
TOR III	(Deamino hydroxy)-TOR	-OH	H
TOR VI	4-OH-(Deamino hydroxy)-TOR	-OH	OH
TOR X	Di-demethyl-TOR	$-NH_2$	H
TOR IV	4-OH-DMTOR	NH-CH_3	OH

N-oxidative demethylation into DMTOR and secondarily by side chain deamination and hydroxylation into TOR III. Altogether, 20 metabolites have been identified in faeces. Several of these metabolites were biologically active [7]. Thus, the present study was undertaken to determine the metabolic pathways of TOR in human liver microsomes and the nature of enzyme(s) involved in the main pathways. In this paper, evidence is presented that human CYP 3A [8] enzyme family*, previously identified as NIF, cyclosporin, erythromycin, TST, E2 and TAM oxidases, is the major enzyme involved in the N-demethylation and deamination-hydroxylation of TOR.

MATERIALS AND METHODS

Chemicals

TOR and all its metabolites were obtained from Orion-Farmos Corporation (Turku, Finland). Caffeine, cimetidine, erythromycin, TAO, TST, lidocaine, E2 and α-NF were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). CsA was supplied by Sandoz (Rueil-Malmaison, France), NIF by Bayer-Pharma (Sens, France), diltiazem by Lers-Synthelabo (Meudon-La-Forêt, France) and gestodene by Schering SA (Lys-Lez-Lannoy, France). DDYA was given to us by Dr J.P. Salaün (Université L. Pasteur, Strasbourg, France).

TOR, labeled with tritium in positions 3 and 5 of the *para*-substituted phenyl ring, was synthesized in Orion-Farmos' chemical research group. The synthesis of tritiated TOR was performed by tritiating the respective brominated TOR with Pd/C catalysis. The labile tritium atoms were washed and the stable product was purified by TLC on silicagel 60 (Merck, Darmstadt, Germany) with *n*-butanol/methanol (70/30; v/v) as eluent. Its specific activity was 20 Ci/mmol and its radiochemical purity greater than 99%.

Human liver samples and microsome preparation

Human liver samples were obtained from 32 subjects: 28 adult kidney donors (27 males, 5 females; mean age: 39 ± 12 years old) who died after traffic accidents and 4 children (Br023, Br024, Br040, see Table 2). Sampling was made in accordance with French legal consideration. Ethical committee approval was obtained prior to this study. At brain death, the liver was removed, frozen immediately and stored in liquid nitrogen until use for preparation of microsomes. Although pre-death dietary and exposure to drugs were not known, P450 specific content in human liver microsomes (Table 2) was in agreement with data previously published [9]. Microsomal fractions were prepared as previously described [10] and stored at −80° until use.

Determination of monooxygenase activities

TOR metabolism. Incubations were carried out in siliconized glass tubes in the dark. The standard incubation mixture contained, in a final volume of 0.5 mL, 100 mM potassium phosphate buffer pH 7.4, 3 mM $MgCl_2$, 0.5 mM TOR and 1 mg of microsomal protein. After 2 min of preincubation at 37°, the reaction was started by addition of 1.2 mM NADPH.

* P450, cytochrome P450 (EC 1.14.14.1); the updated recommended nomenclature for P450 enzymes is according to Nelson *et al.* [8]. The name cytochrome has been abandoned according to the Nomenclature Committee of the International Union of Biochemistry Nomenclature of the electron-transfer proteins; the appropriate name being heme-thiolate protein.

Table 2. P450 dependent activities in microsomal samples from human livers

Subjects	Sex, Age	Total P450‡	CYP3A§	Tam*	TST*	E2*	NIF†	Erythro†	DMTOR*	4-OH-TOR*	TOR III* (a)
FH 1	M, 18y	269	1.2	79	815	26	0.60	1.14	31	5	17
FH 2A	M, 41y	527	5.6	446	4075	394	3.85	2.58	212	30	24
FH 3	M, 47y	470	3.6	151	3420	213	3.37	1.86	139	28	11
Br015	M, 43y	414	2.8	88	985	25	0.97	0.85	30	5	
Br016	M, 44y	369	2.9	123	1850	85	1.67	1.15	40	8	
Br017	M, 26y	593	2.6	164	1795	83	1.78	1.42	70	12	
Br018	F, 45y	334	1.8	93	950	32	0.61	1.00	30	6	
Br019A	M, 45y	241	1.1	37	740	52	1.17	0.82	30	8	
Br021	M, 49y	257	1.2	104	1105	51	0.79	1.06	31	7	
Br022	M, 23y	286	3.3	225	3650	236	5.26	1.90	88	22	12
Br023	M, 5m	228	2.0	97	375	208	0.21	ND	ND	ND	
Br024	F, 15y	505	4.5	299	3935	385	4.14	2.12	146	30	12
Br025	M, 2y	110	1.2	128	1085	76	0.22	0.98	27	6	
Br027	M, 21y	100	1.0	54	435	37	0.31	0.76	27	8	
Br028	M, 27y	165	1.9	100	845	250	1.55	0.50	30	9	
Br029	M, 23y	77	0.7	66	165	49	0.41	0.35	8	1	
Br031	M, 36y	154	0.4	51	430	96	0.22	0.17	21	4	
Br032	M, 44y	286	2.9	134	2445	459	5.98	1.66	192	29	18
Br033	M, 32y	363	1.5	92	1626	196	2.15	0.74	107	16	14
Br034	M, 33y	286	1.3	72	747	92	0.65	0.44	33	7	
Br035	M, 56y	252	2.4	172	2120	565	3.68	1.57	102	20	15
Br036	M, 56y	153	1.1	147	1310	194	1.67	0.64	56	10	
Br037	F, 51y	230	0.9	117	279	17	0.39	0.32	12	6	
Br038	F, 46y	120	1.8	44	324	10	0.35	0.24	12	4	
Br039	M, 60y	538	3.5	328	2450	124	4.47	0.98	114	24	11
Br040	M, 2d	264	1.2	111	30.3	41	0.81	0.36	25	5	
Br041	M, 47y	55	3.0	63	ND	17	0.77	0.94	ND	ND	
Br042	M, 60y	143	3.2	345	ND	367	2.87	2.18	96	21	
Br043	M, 40y	198	2.4	315	3.5	370	2.38	1.18	122	20	
Br046	M, 40y	433	2.2	156	ND	530	2.11	1.06	75	16	
Br047	F, 40y	747	2.7	179	ND	779	2.75	ND	152	23	16
Br048	M, 38y	769	1.6	92	ND	52	0.67	ND	21	8	
Mean	39y(x)	310		146	1407	191	1.84	1.07	71	13	15
±SD	12	184		100	1218	195	1.61	0.63	56	9	4

* pmol/min/mg microsomal protein.
† nmol/min/mg microsomal protein.
‡ pmol/mg microsomal protein (measured by spectrophotometry).
§ arbitrary relative units by mg of proteins.
Age: y = year; m = month; d = day; (x) not including children.
Tam, tamoxifen N-demethylation; TST, testosterone 6β-hydroxylation; E2, estradiol 2-hydroxylation; NIF, nifedipine oxidation; Erythro, erythromycin N-demethylation; DMTOR, toremifene N-demethylation; 4-OH-TOR, toremifene 4-hydroxylation; (a) TOR III, toremifene deamination–hydroxylation (measured by radiolabeling on 10 selected samples).

After 60 min shaking at 37°, the reaction was stopped by addition of 5 mL chilled chloroform. The mixture was vortexed and re-extracted at pH 9.0 with 5 mL of chloroform. The organic extracts were pooled and dried at 40° under nitrogen stream. To the dried residue was added 0.2 mL methanol/water (85/15; v/v) mixture for HPLC analysis. Control incubations were run as described above except that microsomal proteins or NADPH were omitted.

The HPLC analyses of metabolites were performed on a Lichrosorb RP-Select B column (Merck, Darmstadt, Germany) 250 × 4 mm, particle diameter 5 μm, eluted by a mobile phase consisting of methanol, water and triethylamine (80/20/0.1; by vol.) with a flow rate of 0.8 mL/min. Eluates were detected by UV at 238 nm with a sensitivity of 0.005 AUFS. Peaks were identified by their retention times and UV characteristics in comparison with standard compounds as described previously [11]. Peaks were quantified by the integration microcomputer system from Kontron (Kontron Instruments, Saint-Quentin-en-Yvelines, France). Overall biotransformation was calculated by calibrating with known amounts of metabolites. Linearity of DMTOR, 4-OH-TOR and TOR III productions was checked by varying protein concentration (0.5–2 mg) and time (15–60 min).

For the validation of measurement of DMTOR, 4-OH-TOR and TOR III formed from TOR, 10 samples were selected (FH1, FH2A, FH3, Br022, Br024, Br032, Br033, Br039, Br035 and Br047) and were incubated with [^{3}H]TOR 0.5 mM (sp. act. 6 mCi/mmol) as described above. The chloroformic extracts were analysed by HPLC and eluates were collected and their radioactivity counted by liquid scintillation spectrometry.

Identification of TOR metabolites

For the identification of metabolites of TOR, ^{3}H-labeled TOR (sp. act. 6 mCi/mmol) was incubated

at 500 μM concentration with 2 mg of microsomal proteins from Br032, Br039 and Br047 samples for 100 min in the conditions described above. The chloroformic extracts were analysed by HPLC according to two analytical conditions [11] in order to establish the identity of metabolites. The first HPLC analysis was performed as described above. The second HPLC analysis was performed on a Nova-Pak-C18, 4 μm (150 × 3.9 mm) from Waters-Millipore (Milford, MA, U.S.A.), eluted by a mobile phase consisting of ammonium acetate 0.1 M/acetonitrile/triethylamine (54/46/0.5; by vol.) mixture buffered at pH 6.4 by acetic acid. Metabolites were detected by UV at 238 and 277 nm, respectively, for these two columns. HPLC eluates were collected and their radioactivity counted by liquid scintillation spectrometry. Metabolites were identified on the basis of their retention times, UV spectral characteristics, ^{3}H-label and fluorescence detection after photocyclization of eluates [11]. Control incubations were run as described above except that NADPH was omitted.

Other monooxygenase activities

TST 6β-hydroxylation, erythromycin N-demethylation, TAM N-demethylation, estradiol 2-hydroxylation, NIF oxidation activities were measured according to procedures previously published [12, 13].

Inhibition of TOR metabolism by different compounds

Incubations were performed as described above with 500 or 100 μM TOR in 5 μL DMSO. Compounds such as diltiazen, TAO, lidocaine, cimetidine, caffeine, CsA, 17α-E-E2 were previously added in 5 μL methanol or DMSO at concentrations ranging between 10 and 500 μM. Addition of these organic solvents did not inhibit DMTOR formation. Control experiments were conducted with the same amounts of organic solvent. The FH2A, FH3 and Br047 liver samples were used for these experiments; they were chosen on the basis of their high TOR metabolizing activities.

Inhibition of TOR demethylation by gestodene or 11-dodecynoic acid, two suicide substrates [14, 15], was carried out as described by Guengerich [14]. In outline, the initial incubation was performed with 1 mg of microsomal protein, 100 μM gestodene and 5 mM NADPH for 30 min at 37°. The 0.1 mL incubation medium was then diluted 5-fold into 0.1 mM potassium phosphate buffer pH 7.4 containing 0.5 mM TOR and 1 mM NADPH. After 60 min incubation at 37°, the metabolites were extracted and measured by HPLC as described above. For inhibition of TOR metabolism by DDYA, the initial incubation was performed with 1 mg of microsomal protein, 0–200 μM DDYA added in methanol/water (50/50; v/v), 3 mM $MgCl_2$ and 5 mM NADPH for 30 min at 37°. To 0.47 mL incubation medium was added 0.5 mM TOR and 1 mM NADPH. After 60 min incubation at 37°, the metabolites were analysed as described above. Control experiments were conducted according to the same procedure with the same amounts of organic solvent (never exceeding 2%, v/v).

Immunoblot analysis

Protein samples (20 μg) were separated by electrophoresis on 9% SDS–polyacrylamide gels according to Laemmli [16] and transferred electrophoretically to a nitrocellulose sheet [17]. After incubation at 37° for 30 min by 3% bovine serum albumin and 10% newborn calf serum in PBS, the nitrocellulose sheet was sequentially treated with human monoclonal anti-P450-NIF [18] overnight at 4°, washed with PBS then PBS containing rabbit anti-mouse immunoglobulins conjugated to peroxidase (Dako, Versailles, France). Finally the sheet was washed with PBS and the peroxidase activity, was detected with 4-chloronaphthol and H_2O_2. The quantification of P450 3A was performed by means of image processing Scan analysis (Biosoft, Cambridge, U.K.). The integrated blot area of the various microsomal preparations was expressed as arbitrary units relative to the amount of proteins.

Immuno-inhibition of TOR metabolism

Microsomal preparations from FH3 sample containing 750 pmol of total P450 in 100 mM potassium phosphate buffer pH 7.4, 3 mM $MgCl_2$ in the absence of TOR and NADPH were incubated at room temperature for 30 min in the presence of increasing amounts (1–10 mg IgG/nmol P450) of polyclonal P450 3A antibody or non-immune rabbit IgG. Reaction was started by 0.5 mM TOR and NADPH additions and processed as described above. The antibody CYP3A4 was raised against human CYP3A4 expressed in *Escherichia coli* (Belloc C, unpublished results). The metabolites were analysed as described above.

cDNA expression of different CYP in yeasts

CYP1A1/1A2, CYP2C9 coding sequences were cloned by polymerase chain reaction from human liver cDNA templates and after sequencing them for control, they were inserted in the yeast expression vector YeDP60 [19]. Human CYP3A4 coding sequence was inserted in YeDP60 by gap repair [20]. Each expression vector was introduced into the engineered *Saccharomyces cerevisiae* strain W(R) which over-expresses yeast NADPH-P450 reductase when grown with galactose as carbon source [21]. Culture medium S5 (containing galactose) without adenine, spheroplast preparation by enzymatic digestion of yeast cell walls and sub-cellular fractionation were as described previously [20, 21].

Statistical analysis

Correlation coefficients were calculated using an ANOVA table by the least squares regression analysis from the raw data. As a quite normal gaussian distribution in the population was observed (skewness = 0.89 for DMTOR formation), correlation coefficients were calculated by including all the samples. Correlation coefficients were considered to be statistically significant when P was <0.005.

RESULTS

Metabolites of TOR

By using the optimized HPLC conditions, especially a column-Lichrosorb RP-Select B (specific

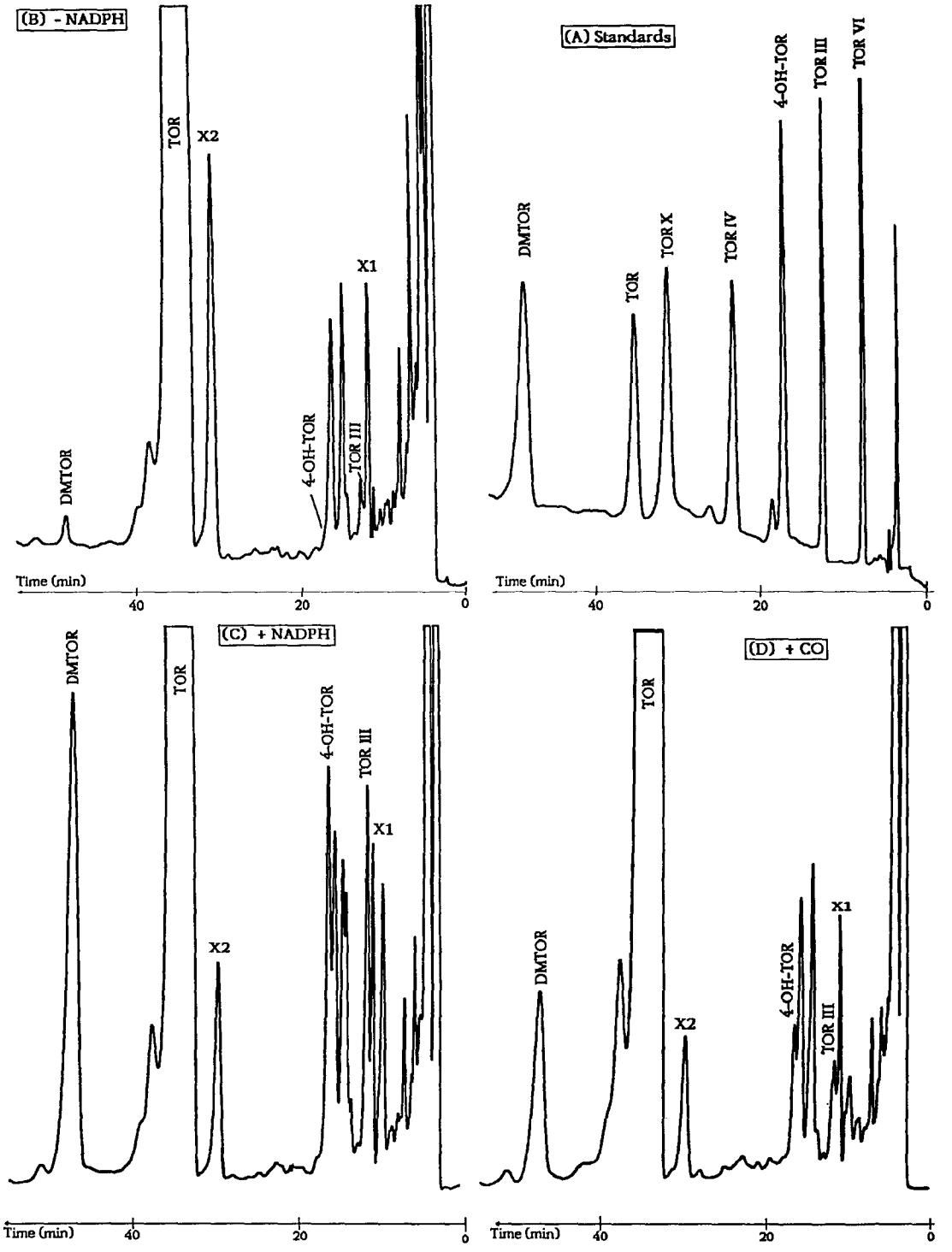


Fig. 1. HPLC chromatograms of TOR metabolism. (A) Pure compounds; (B) chromatogram of Br032 sample (1 mg microsomal protein) incubated with 0.5 mM TOR without NADPH; (C) and (D) chromatograms of incubation medium of 0.5 mM TOR with 1 mg of microsomal sample from Br032 human liver containing 1 mM NADPH (C) or containing NADPH but bubbled with CO (D). For identification of peaks, see Table 1; X1 and X2 unknown compounds. Analysis was carried out on a Lichrosorb RP-Select B 5 μm Column (25 × 0.4 cm); mobile phase was methanol/water/triethylamine in the proportions (80/20/0.1; by vol.) at a flow rate of 0.8 mL/min. Eluates were detected at 238 nm with 0.005 AUFS.

for basic compounds) seven compounds presented in Table 1 were separated within 65 min (Fig. 1). The specificity of the method is based upon the chromatographic behavior of TOR metabolites according to stationary phase. On the Lichrosorb RP-Select B stationary phase, basic compounds such as DMTOR, TOR or TOR X were the most retained while on standard octadecylsilane column such as Nova-Pak C18 phase, polar compounds such as TOR III without amino group were the most retained [11].

Three main metabolites of TOR were identified when incubated with human liver microsomal preparations: *N*-demethyl-TOR (TOR I), 4-OH-TOR (TOR II) and TOR III. The identity of these metabolites was based upon their radiolabeling, their retention times and UV characteristics (Fig. 1). This figure shows that UV detection could not always provide sufficient discrimination to determine the amount of minor metabolites such as 4-OH-TOR and TOR III, particularly when the rate of formation of products was low. So, in order to validate the amount of formed metabolites, two experiments were conducted. In a first experiment, [^{3}H]TOR was incubated with 10 selected samples and the metabolites separated on the two HPLC phases, described above, were collected and their radioactivity counted. The metabolic rates determined either by UV or radioactivity measurements were highly correlated ($r > 0.95$, for the three metabolites, data not shown). In a second experiment, the HPLC eluates were UV-photocyclized and detected by spectrofluorimetry as described previously [11]. Results were quite similar whatever the mode of detection for the three main metabolites (data not shown). Unlike TAM, the *N*-oxide metabolite of TOR could not be detected.

Preliminary experiments suggested the involvement of cytochrome P450 in TOR metabolism by human liver microsomal samples. This evidence included localization of activity in the microsomal subcellular fraction, dependence upon the presence of NADPH for catalytic activity (Fig. 1C). However, in the absence of supplemented NADPH, there was a trace amount of *N*-desmethyl metabolite formed (Fig. 1B). Moreover, DMTOR, 4-OH-DMTOR and TOR III formations from TOR were inhibited up to 79 ± 5, 72 ± 3 and $79 \pm 5\%$, $N = 5$ samples, respectively vs control when the medium reaction was bubbled with a CO/O_2 (80/20; v/v) mixture before starting reaction (Fig. 1D).

Kinetic parameters

Figure 2 shows the double reciprocal plots of DMTOR, 4-OH-TOR and TOR III formations from TOR by microsomes from human liver Br047. K_m was determined as 124 μM with a V_m of 142 pmol/min/mg microsomal protein for DMTOR formation. K_m was determined 139 μM with a V_m of 20 pmol/min/mg microsomal protein for 4-OH-TOR formation. Apparent K_m was 16 μM for TOR III formation.

Correlations between TOR metabolism and different monooxygenase activities in human liver microsomes

Table 2 shows the rate of biotransformation of

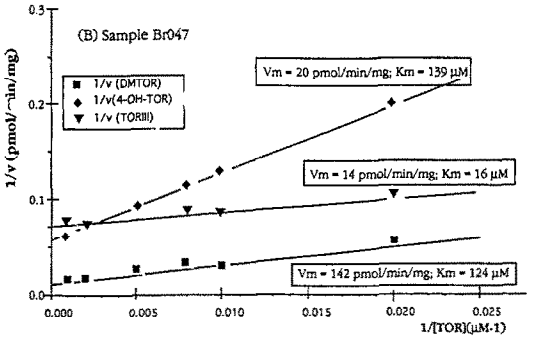


Fig. 2. Reciprocal plot of v (pmol/min/mg protein) against TOR (μM^{-1}) metabolism according to Lineweaver–Burk. Microsomal preparations from Br047 sample were studied: 1 mg of microsomal protein was incubated for 60 min with concentrations of TOR ranging from 50 to 1000 μM added in 5 μL of DMSO. (■) DMTOR formation; (◆) 4-OH-TOR formation; (▼) TOR III formation.

TOR, TAM, TST, NIF and erythromycin by 32 human liver microsomes. The rates of N-oxidative demethylation and 4-hydroxylation of TOR ranged from 8 to 212 and from 1 to 30 pmol/min/mg with means of 71 ± 56 and 13 ± 9, respectively. The rate of TOR III formation measured with only 10 selected human liver microsomal preparations by means of [^{3}H]TOR incubation was 15 ± 4 pmol/min/mg protein.

If two reactions are catalysed by the same enzyme, then the rates should be correlated to each other when compared in a series of microsomal preparations containing varying levels of the enzyme. The N-oxidative demethylation of TOR correlated significantly with five catalytic activities known to be supported by CYP3A4 enzyme, namely NIF oxidation ($r = 0.86$), erythromycin N-demethylation ($r = 0.80$), TST 6β-hydroxylation ($r = 0.78$), E2 2-hydroxylation ($r = 0.75$), TAM *N*-demethylation ($r = 0.73$) (Table 3). In no case, was an r value >0.35 found for substrates marker of CYP1A2 (phenacetin-O-deethylation; methoxy- or ethoxy-resorurfin O-dealkylations, acetanilide 4-hydroxylation or caffeine demethylations measured according to [10]) or CYP2E1 (*N*-nitroso-dimethylamine demethylation, butanol oxidation or chlorzoxazone 6-hydroxylation measured according to [22]). The conclusion was reached that none of these P450s make a major contribution to TOR metabolism. However, a significant correlation but weaker was observed for coumarin 7-hydroxylation specific to CYP2A6. On the contrary, TOR III formation was significantly correlated with monooxygenase activities specific to CYP1A enzymes (caffeine demethylations, acetanilide 4-hydroxylation) and CYP2E1 (nitroso-dimethylamine demethylation, butanol oxidation and chlorzoxazone 6-hydroxylation).

Correlation of TOR biotransformation with immunoquantified CYP3A4

The N-oxidative demethylation (Fig. 3) of TOR

Table 3. Correlation coefficients (r) between catalytic activities and N-oxidative demethylation (DMTOR), 4-OH-hydroxylation (4-OH-TOR) and demethylation plus hydroxylation (TOR III) of TOR in human liver microsomal samples

Number of subjects	Monooxygenase activity	DMTOR	4-OH-TOR	TOR III	Tamoxifen N-demethylation	Testosterone 6β-hydroxylation	Nifedipine-oxidation	Erythromycin *N*-demethylation	Estradiol 2-hydroxylation	Caffeine demethylations	Phenacetine O-deethylation	Acetanilide 4-hydroxylation	Ethoxy-Resorufin-O-Deethylation	Methoxy-Resorufin-O-Deethylation	Nitrosomethylamine demethylation	Butanol oxidation	Chlorzoxazone 6-hydroxylation	Coumarin 7-hydroxylation
30	DMTOR																	
30	4-OH-TOR	**0.96**																
10	TOR III	0.45	0.05															
32	Tamoxifen N-demethylation	**0.73**	**0.77**	0.28														
27	Testosterone 6β-hydroxylation	**0.78**	**0.85**	0.01	**0.67**													
32	Nifedipine-oxidation	**0.86**	**0.92**	0.09	**0.69**	**0.86**												
29	Erythromycin *N*-demethylation	**0.80**	**0.83**	0.39	**0.70**	**0.92**	**0.74**											
32	Estradiol 2-hydroxylation	**0.75**	**0.73**	0.29	**0.63**	**0.66**	**0.70**	**0.62**										
26	Caffeine demethylations	0.40	0.27	**0.73**	0.37	0.37	0.19	0.39	0.15									
29	Phenacetine O-deethylation	0.24	0.16	*0.48*	0.31	0.11	0.05	0.30	0.14	**0.74**								
27	Acetanilide 4-hydroxylation	0.20	0.10	**0.75**	0.18	0.15	0.09	0.19	0.24	**0.78**	**0.63**							
29	Ethoxy-Resorufin-O-Deethylation	0.33	0.25	*0.56*	0.43	0.19	0.20	0.19	**0.73**	**0.70**	**0.64**	**0.71**						
29	Methoxy-Resorufin-O-Deethylation	0.26	0.05	0.33	0.26	0.08	0.04	0.08	0.08	0.46	**0.71**	0.46	**0.89**					
23	Nitrosomethylamine demethylation	0.07	0.26	**0.64**	0.02	0.21	0.12	0.12	0.10	0.09	0.17	0.00	0.13	0.05				
23	Butanol oxidation	0.17	0.17	*0.55*	0.06	0.26	0.19	0.27	0.24	0.03	0.23	0.13	0.11	0.04	**0.87**			
29	Chlorzoxazone 6-hydroxylation	0.16	0.07	*0.54*	0.04	0.15	0.17	0.12	0.17	0.14	0.02	0.17	0.08	0.05	**0.76**	**0.72**		
28	Coumarin 7-hydroxylation	**0.62**	*0.48*	0.32	*0.48*	0.18	0.46	0.41	*0.59*	0.15	0.38	0.21	0.47	0.59	0.25	0.23	0.37	

Correlation coefficients (r) were calculated by the least squares method. For $r > 0.50$, $P < 0.01$ for $N > 25$ (r in italics) and for $r > 0.65$, $P < 0.005$ for $N > 25$ (r in bold).

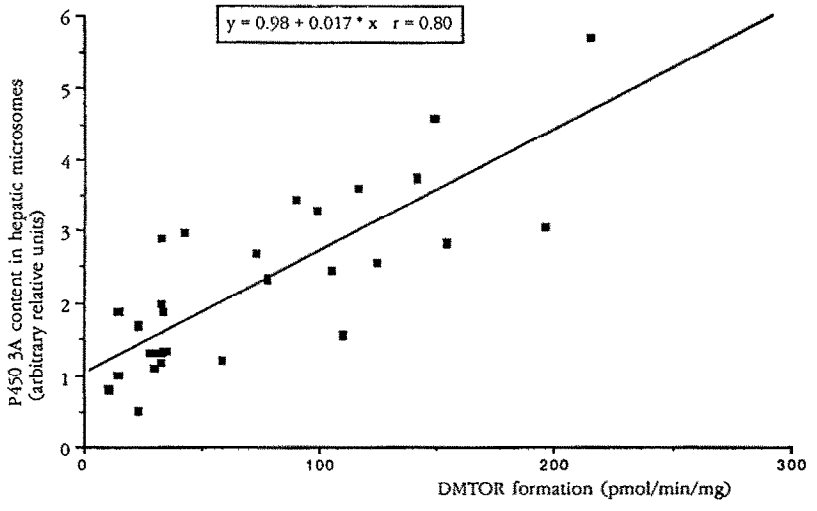


Fig. 3. Correlation between N-oxidative demethylation of TOR and immuno-quantitated CYP3A enzymes immunodetected in human liver microsomes. Intensity units of CYP3A are relative arbitrary units determined by densitometry. Correlation coefficient (r) was calculated by the least-squares regression method. The equation of the regression line was: $y = 0.98 + 0.017^*x$, $r = 0.80$.

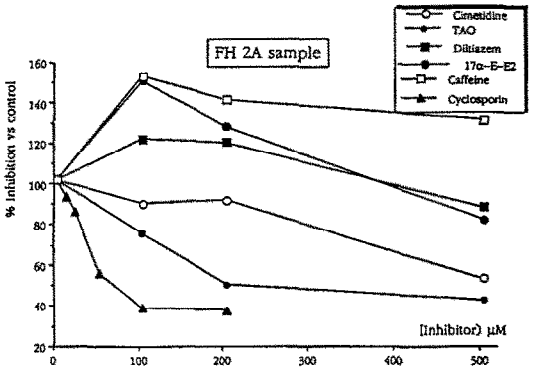


Fig. 4. Effect of different drugs on the N-oxidative demethylation of TOR by the microsomal preparation of FH2A liver by different drugs. (○) Cimetidine; (◆) TAO; (■) diltiazem; (●) 17α-E-E2; (▲) CsA; (●) caffeine.

correlated significantly with the amount of CYP3A immuno-detected by a monoclonal antibody anti-human CYP3A4 ($r = 0.80$; $N = 30$; $P < 0.001$) by means of western blot technique. The regression line failed to intersect the y axis at zero, suggesting the involvement of other P450 isozymes in the N-demethylation of TOR. No significant correlation could be measured between DMTOR and 4-OH-TOR formations and the amount of CYP1A2 ($r = 0.30$ and 0.17; $N = 24$) and CYP2E1 ($r = 0.04$ and 0.08; $N = 24$)—results not shown.

Inhibition of TOR metabolism by different compounds

To assess further whether or not the different metabolic pathways of TOR are catalysed by the CYP3A enzymes, the inhibitory effect of different substrates of P450 was tested on the FH2A microsomal sample with one relatively high substrate concentration, i.e. 500 µM (Fig. 4). A weak inhibition of N-demethylation of TOR was observed when the microsomes were incubated with some selective inhibitors of other human liver P450s, such as caffeine. In contrast, compounds known to be metabolized by CYP3A, namely cyclosporin, TAO, 17α-E-E2, diltiazem and cimetidine inhibited the N-demethylation of TOR at 60 ± 6, 50 ± 12, 47 ± 2, 39 ± 17 and 47 ± 2% (N = 3 samples: FH2A, FH3, Br047) vs control, respectively, at inhibitor concentration of 200 µM. The 4-hydroxylation of TOR was only weakly inhibited by cimetidine (18 ± 10%), TAO (37 ± 20%), diltiazem (33 ± 8%), 17α-E-E2 (42 ± 11%) and caffeine (15 ± 5%) vs control for the same microsomal samples. Lidocaine 1 mM inhibited the 4-hydroxylation and N-demethylation of TOR no more than 27 ± 11 and 39 ± 14%, respectively, in three samples (FH3, Br032 and Br042). These results could suggest that about 40 to 50% of the conversion of TOR to DMTOR and 4-OH-TOR was not mainly metabolized by CYP3A enzymes. This is unlike the metabolism of TAM which is very nearly inhibited by drugs such as TAO or CsA [12]. Therefore, inhibition assays of TOR metabolism were performed using a lower substrate concentration (100 µM) than previously used. Results reported on Table 4 show that CsA and TAO inhibited the formation of DMTOR and TOR III by more than 75% while the formation of 4-OH-TOR was inhibited by no more than 50%. The inhibitory effect of TST was more complex. If the formation of TOR III was inhibited at about 75%, this steroid inhibited the N-demethylation and 4-hydroxylation of TOR by no more than 50%. It is noteworthy that the inhibition was less efficient in the Br047 sample than in the two other samples (Table 4).

It could be suspected that α-naphthoflavone might enhance the TOR metabolism because some CYP3A4-catalysed reactions are stimulated by this compound [23–25]. However, increasing concentrations of α-NF up to 50 µM did not activate the TOR metabolism but inhibited it about 60% (results not shown). This result is in agreement with previous data reporting that some reactions can be inhibited by α-NF although they are CYP3A4-mediated [26].

Surprising, however, is our observation that estradiol did not significantly inhibit the N-demethylation pathway (12% inhibition with 0.1 mM estradiol) because this steroid is a substrate for CYP3A in human liver microsomes [13]. Similar results were previously reported concerning TAM [12].

Inhibition of TOR metabolism by gestodene and DDYA

Gestodene, a 17α-acetylenic steroid, is known to

Table 4. Percentage inhibition (vs control) of formation of three metabolites of TOR 0.1 mM by CsA, TAO and TST in three microsomal human liver samples FH-2A, FH-3 and Br047

Human liver sample	TOR III			4-OH-TOR			DMTOR		
Inhibitor	CsA	TAO	TST	CsA	TAO	TST	CsA	TAO	TST
FH-2A	76	87	80	47	48	55	78	92	45
Br047	72	72	59	51	51	21	82	85	39
FH-3	70	88	80	57	70	63	81	90	54

TOR 0.1 mM was incubated with 1 mg of microsomal proteins and 1 mM NADPH in the presence of CsA and TAO added at 0.1 mM or TST added at 0.2 mM for 1 hr at 37°. Metabolites were extracted then separated by HPLC on Lichrosorb RP-Select B as described in Materials and Methods. Control values for TOR III, 4-OH-TOR and DMTOR were 20, 25, and 133 for FH-2A sample, 11, 15 and 118 for FH-3 sample and 10, 13 and 79 pmol/min/mg protein for Br047 sample, respectively.

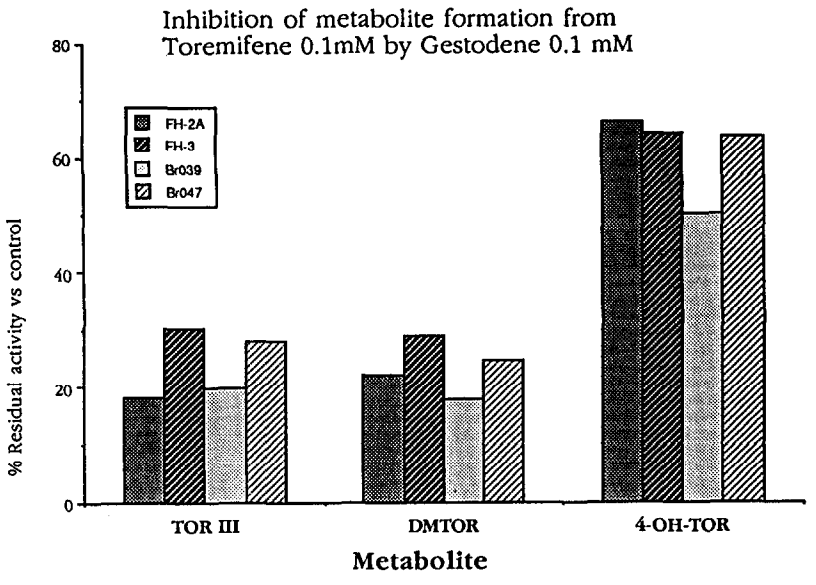


Fig. 5. Inhibition of DMTOR, TOR III and 4-OH-TOR formations from 0.1 mM TOR by 100 μM gestodene in four microsomal liver samples FH2A, FH3, Br039 and Br047. The preincubation was carried out with 5 mM NADPH and 100 μM gestodene with 1 mg microsomal protein for 30 min. This step was followed by incubating 100 μM TOR for 60 min as described in Materials and Methods.

be a mechanism-based inactivator of CYP3A4 [14]. This inactivation process is postulated to result from attack of P450 on the substituted carbon of the acetylene and leads to heme modification. Preincubation of four microsomal samples (Fig. 5) with 100 μM gestodene dramatically reduced the N-oxidative demethylation [mean residual activity: 23.5 ± 4.6% (±SD) vs control] and TOR III formation (mean residual activity: 24.1 ± 6% (±SD) vs control while the 4-hydroxylation of TOR was less inhibited [mean residual activity: 60.9 ± 7.3% (±SD)]. The N-oxidative demethylation and 4-hydroxylation of TOR were inhibited by no more than 53 ± 10.9 and 58.9 ± 9.4% vs control, respectively, when TOR was incubated at a concentration of 0.5 mM (results not shown).

On the other hand, another acetylenic compound, 11-dodecynoic acid, preincubated 30 min with the microsomal sample FH2A did not inhibit the TOR metabolism: at 200 μM the inhibition was only 8 ± 2%.

Metabolism of TOR by microsomes from yeasts expressing human P4501A1, 1A2, 2C9 and 3A4

Incubation of TOR with the microsomal preparations of yeast cells that were genetically engineered for stable expression of human P4501A1, 1A2, 2C9 and 3A4 resulted in the formation of two metabolites, namely DMTOR and TOR III. In no case, could 4-OH-TOR be detected. Results expressed as metabolic rate or turnover number in min^{-1} are reported on Table 5. P4501A1 is the major enzyme involved in the formation of DMTOR and TOR III, at least at a substrate concentration of 0.5 mM. The contribution of P450 3A4 is clearly established in the N-demethylation and the side-chain deamination and subsequent hydroxylation.

Immuno-inhibition of TOR metabolism

In order to confirm that CYP3A enzymes are involved in TOR metabolism, immuno-inhibition studies with anti-human CYP3A4 were carried out.

Table 5. Metabolism of TOR by microsomes from yeasts expressing human P450s

Yeast strains	DMTOR formation (pmol/min/mg)	DMTOR turnover number (min^{-1})	TOR III formation (pmol/min/mg)	TOR III turnover number (min^{-1})
1A1 W(R)	24.1	2.1	2.5	0.22
1A2 W(R)	8.6	0.30	0.92	0.031
2C9 W(R)	0.30	0.04	0	0
3A4 W(R)	11.9	0.52	1.2	0.053

One milligram of microsomal proteins from engineered yeasts was incubated with 0.5 mM TOR for 60 min. Metabolites, after extraction, were separated by HPLC and detected by UV at 237 nm and by fluorescence after UV photooxidation as described previously [11]

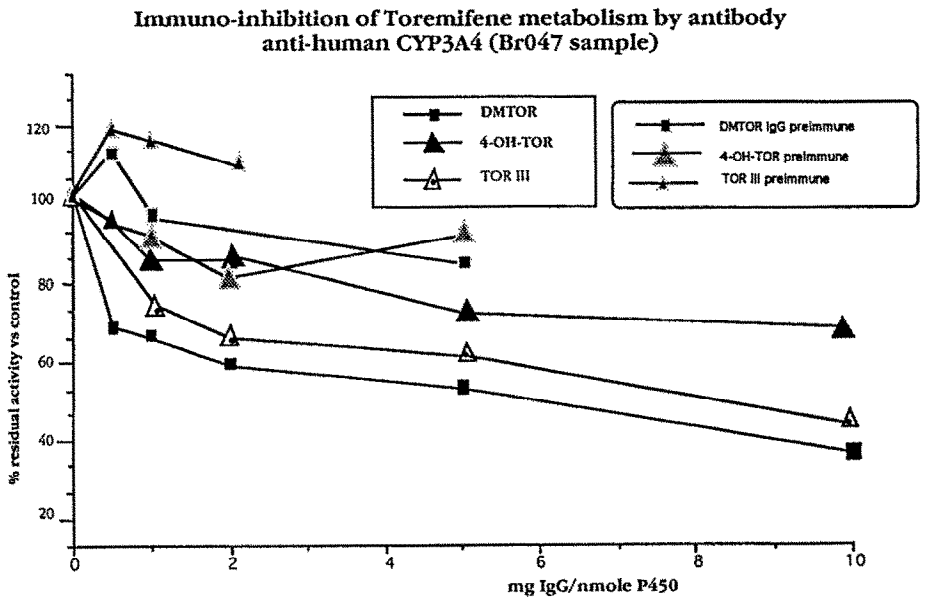


Fig. 6. Effect of anti-CYP3A4 antibody on N-oxidative demethylation [(■) DMTOR formation], 4-hydroxylation [(▲) 4-OH-TOR formation] and TOR III (△) formation. Microsomes from sample FH3 were preincubated with preimmune IgG (dotted line) or anti-CYP3A4 IgG (full line). One milligram of microsomal protein from Br047 sample (750 pmol of total P450) incubated with 0.5 mM TOR for 60 min after preincubation for 30 min at room temperature with anti-CYP3A IgG. Reaction was started with addition of TOR and NADPH. Control experiments were performed with rabbit preimmune IgG.

This study showed that this antibody was inhibitory of two metabolic pathways, namely N-oxidative demethylation—60% inhibition, and TOR III formation—46% inhibition (Fig. 6). However, the extent of inhibition of N-demethylation was widely variable in individual livers (58, 36 and 48% with 2 mg IgG/nnol P450 for FH3, Br042 and Br047 samples, respectively).

DISCUSSION

Structurally, TOR is very similar to TAM, with the exception of a chloride atom substitution for a hydrogen atom on the ethylene alkyl side chain of TAM. Thus, the major metabolites of TOR are also structurally similar to the metabolites of TAM. TOR is extensively metabolized *in vivo*, and over 20 metabolites have been identified, mostly in faeces [1–3]. However, the rate of metabolism of TOR by human liver microsomes was markedly lower than that of TAM (Table 2). Two major metabolites, namely DMTOR and 4-OH-TOR, are probably active [7] and the N-oxidative demethylation is the major route of TOR metabolism. In humans treated with TOR, plasmatic DMTOR is present at concentrations up to four times higher than TOR steady-state concentrations [3, 27]. In contrast, 4-OH-TOR plasma concentration are only 30% of the TOR levels [27]. The present study demonstrates that these two metabolites are mainly formed into the liver by cytochrome P450. These two enzymatic reactions were inhibited by carbon monooxide and were NADPH dependent. If the N-demethylation of both TOR and TAM appears to represent the major primary metabolic pathway of TAM and TOR, the 4-hydroxylation represents about 1/5 (0.22 ± 0.08; $N = 30$) of that of DMTOR in human liver microsomes whereas this 4-hydroxylation was a very minor pathway in TAM metabolism [11, 28]. Such a result is in agreement with previous data reporting that the 4-hydroxylation was more important for TOR than TAM [27]. In contrast, the metabolic conversion of TOR into deaminohydroxy-TOR appears to have been subject to controversy as it is not a metabolite of TAM. While some authors [1–3, 29] detected TOR III in human plasma at concentrations of about 1/10 of that of parent drug, other authors were not able to detect this metabolite [27]. Our study shows that the microsomal preparations of human livers metabolized TOR into deaminohydroxy-TOR by side chain deamination and subsequent hydroxylation. The identity of such a metabolite was confirmed by its chromatographic behavior on two different HPLC columns giving very different retention times (relative retention times vs TOR: 0.30 and 1.26 on Lichrosorb RP-Select-B phase and Nova-Pak C-18 phase, respectively). Moreover, by means of incubation of [^{3}H]TOR, this metabolite was shown to be radiolabeled. The TOR III formation was shown to be cytochrome P450-dependent because it needed NADPH and was inhibited by carbon monooxide, and classical P450 inhibitors. The *N*-oxide metabolite of TOR was not detectable while it represented about half of the *N*-demethyl metabolite of TAM in human liver microsomes [12, 28]

This study, using several different approaches suggests that human hepatic TOR N-demethylation is mediated mainly by CYP3A enzyme family. Indeed, a significant correlation between these two activities and TAM *N*-demethylase [12], TST, 6β-hydroxylase [12, 30], NIF oxidase [31], erythromycin *N*-demethylase [32] and estradiol 2-hydroxylase [13] activities was observed in the 32 microsomal preparations studied. All these monooxygenase activities are known to be mediated by the CYP3A4 isoform. The extensive inhibition of microsomal metabolite formation by drugs such as cyclosporin [33], TAO [34], diltiazem [35], 17α-E-E2 [36], cimetidine [25] and lidocaine [37] at a concentration of 200 μM provided further supportive evidence of the involvement of CYP3A enzyme. Although TOR III formation was not correlated with these activities probably because of the low number of tested samples, nevertheless strong support that the formation of this metabolite also involved CYP3A enzymes is derived from the following results. Thus, gestodene, known to be a mechanism-based inactivator of CYP3A4 [14] was able to inhibit significantly the TOR N-demethylation and TOR III formations. Furthermore, these two metabolic pathways were significantly inhibited by antibody directed against CYP3A enzymes, although this inhibition varied widely inter-individually. Cyclosporin and TAO were shown to be the most powerful inhibitors of DMTOR and TOR III formations. As TAO has been shown to be as effective and selective inhibitor as polyclonal antibodies directed against CYP3A enzymes [38], this latter result is consistent with the suggestion that CYP 3A enzyme family is involved in the two major metabolic pathways of TOR. The inhibition assays using a TOR concentration close to K_m demonstrated that cyclosporine and TAO were able to inhibit these two pathways by more than 75%. As the TOR plasmatic level was about 2 μM in treated patients [27], drugs metabolized by CYP3A enzymes if administered with TOR could alter the blood levels and hence the therapeutic potential of the anticancer drug. Finally, direct evidence for the involvement of CYP3A enzyme in DMTOR and TOR III formations was provided by using microsomes derived from yeasts engineered for expressing human P4503A4.

What P450 enzyme(s) is involved in the 4-hydroxylation of TOR cannot be assessed by the present study. Although the 4-hydroxylation activity was correlated with many activities supported by P450 3A4, CYP3A enzymes do not appear to take a significant part in this metabolic pathway because its formation was not significantly inhibited by substrates of CYP3A4, or by gestodene and by polyclonal antibody anti-CYP3A. Lastly, this metabolite was not formed from toremifene by P450 3A4 produced by engineered yeasts.

If this study demonstrates that CYP3A is the major enzyme involved in two main metabolic pathways of toremifene at low concentration of TOR, it could not be excluded that other P450s could be involved, at least at a high concentration such as 500 μM. Indeed, the inhibition of TOR metabolism by substrates known to be metabolized by CYP3A4 such as TAO and CsA did not exceed about 50% vs control at substrate concentration of 0.5 mM while this inhibition was by more than 75% at substrate concentration of 0.1 mM. Thus, it can be suggested that multiple forms of P450 could significantly contribute to TOR metabolism according to their relative affinities. In a previous study [12], we have observed that E2 did not inhibit the metabolism of TAM no more than 15%. A similar result was observed for TOR in the present study and has recently been confirmed [28], suggesting that E2 was not a potent inhibitor of CYP3A4 monooxygenase activities. The contribution of CYP1A1/1A2 to TOR metabolism cannot be completely excluded owing the turnover-number of DMTOR and TOR III formations by these enzymes expressed in modified yeasts. As CYP1A1 is not expressed in human liver [39], only CYP1A2 could be involved in these two metabolic pathways, at least in samples characterized by a high level of expression of this isoform.

The CYP 3A subfamily in man appears to be composed of at least four genes, 3A3, 3A4, 3A5 and 3A7. CYP3A3 and 3A4 are so highly related that they can only be differentiated at the mRNA level [40]. Furthermore, CYP3A3 appears to be present at minor levels, if at all. Due to the lack of probes that would be metabolized by specific isoforms of this family [41], the contribution of these different forms to TOR metabolism could not be determined. However, as CYP3A4 is the major subfamily isozyme expressed in all the human livers [42], it can be suggested that it is involved in the major metabolic pathways TOR. The contribution of CYP3A5, which is expressed in 30% of human livers [42] cannot be totally excluded.

The list of drugs and endogeneous compounds which have been characterized as specific substrates of human P450 3A enzymes includes erythromycine, NIF, cyclosporin, TST, diltiazem, cortisol, TAM propafenone and lidocaine (for a complete list of CYP3A4 substrates, see Ref. 43). It can therefore be anticipated that any of these drugs should lead to interaction with TOR when given in association. However, it should be kept in mind that the extent of this effect is likely to be modulated by various factors such as dose, biodisponibility and relative K_m of both drugs. Thus, it has to be emphasized that TOR therapy may be complicated when given in association with drugs known to be inducers of inhibitors of P450 3A enzymes.

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