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Short communication

P450 interaction with farnesyl-protein transferase inhibitors Metabolic stability, inhibitory potency, and P450 binding spectra in human liver microsomes

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Abstract

Methyl substitution at the 2-position of the imidazole ring greatly improved drug metabolism profiles, in human liver microsomes, of *ras* farnesyl-protein transferase inhibitor (FTI) candidates for drug development. Methyl substitution markedly reduced the P450 inhibitory potency of non-substituted FTIs for CYP3A4 (by a factor of 12-403) and 2C9 (by a factor of 4.2-28), while it had little effect on the CYP2D6 enzyme. An immunochemical inhibition study demonstrated that CYP3A4 plays a predominant role in the metabolism of both non-substituted and 2-methyl-substituted imidazole-containing FTI candidates. Very strong type II binding spectra with human liver microsomes were observed for all non-substituted FTIs, while methyl substitution markedly weakened type II spectra or shifted the type of spectra from II to I. This indicated that methyl substitution on the imidazole moiety interfered with the substrate-P450 heme interaction, likely due to a steric effect caused by the methyl group. A kinetics study revealed that the methyl substitution increased V_{max} and K_m values to the same extent. These studies suggested that the 2-methyl substitution on the imidazole ring improved its drug metabolism profile by reducing the potential to inhibit CYP3A4-mediated metabolism without affecting intrinsic metabolic clearance (V_{max}/K_m).

Keywords: Farnesyl-protein transferase inhibitor; CYP3A4; Type II binding; Type I binding; P450 binding spectra; Metabolic inhibition

1. Introduction

Mutational activation of the *ras* gene has been found in many types of human cancers including more than 90% of pancreatic, 50% of colon, and 30% of lung cancers [1,2]. Ras proteins are synthesized as cytosolic precursor molecules and biologically activated after a series of posttranslational modifications including the prenylation reaction, which attaches the farnesyl group to the protein [3]. Therefore, inhibitors of the first step of the farnesylation of a cysteine residue, catalyzed by the enzyme FTase, have been considered as potential targets for the development of antitumor agents [4–6]. Although a number of thiol-containing compounds have been synthesized based on the sequence motif CAAX and have been proven to be effective for the inhibition of ras FTase activity [7–10], the presence of oxidizable thiol functionality conferred disadvantages on their development as therapeutic agents. Therefore, discovery efforts were directed more toward the identification of non-thiol-containing FTIs. Among the non-thiol-containing FTIs, a series of imidazole-containing agents were effective ligands of the active site (zinc) of FTase [6,11–13] and demonstrated inhibitory potency.

Imidazole-containing agents have generally been well known for their potent inhibition of P450 [14,15]. A previous study on rats demonstrated that a 2-methyl substitution on the imidazole ring of one of the typical FTIs greatly increased the metabolic rate (V_{max}) and reduced the potency of the compound to inhibit CYP3A [16]. The purpose of the

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Abbreviations: FTI, farnesyl-protein transferase inhibitor; FTase, farnesyl-protein transferase; and LC-MS, liquid chromatography-mass spectrometry.

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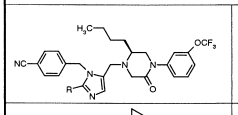
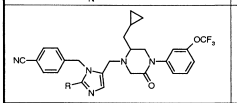
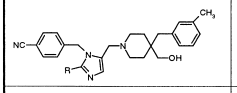
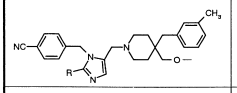
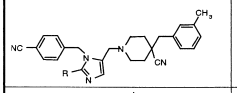
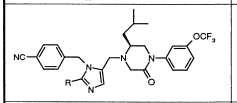
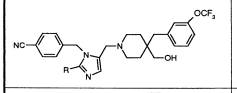
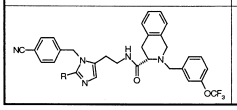
Table 1
P450 inhibition by imidazole- and 2-methylimidazole-containing FTIs in human liver microsomes

Compound		P450 Inhibition (IC_{50}, μM)		
		CYP3A4	CYP2C9	CYP2D6
	R=H **A1** R=CH_3 **A2**	0.10 8.09 (81)[a]	2.38 13.2 (5.6)	25.2 16.0 (0.64)
	R=H **B1** R=CH_3 **B2**	0.25 9.64 (39)	3.89 18.2 (4.7)	15.3 20.2 (1.3)
	R=H **C1** R=CH_3 **C2**	0.24 41.8 (174)	5.85 163 (28)	14.9 18.8 (1.3)
	R=H **D1** R=CH_3 **D2**	0.23 92.7 (403)	2.92 50.9 (17)	39.8 44.0 (1.1)
	R=H **E1** R=CH_3 **E2**	0.23 17.5 (76)	24.0 >100 (>4.2)	32.4 44.3 (1.4)
	R=H **F1** R=CH_3 **F2**	0.15 41.0 (273)	1.75 10.2 (5.8)	15.0 14.3 (0.95)
	R=H **G1** R=CH_3 **G2**	0.89 77.5 (87)	7.83 80.8 (10.3)	18.4 18.8 (1.0)
	R=H **H1** R=CH_3 **H2**	3.31 38.6 (12)	11.9 71.1 (6.0)	13.1 13.6 (1.0)

[a] Numbers in parentheses indicate the ratio of the value for 2-methylimidazole-containing FTI to that for the corresponding non-substituted FTI.

present study was to examine the effect of 2-methyl substitution of the imidazole ring on metabolic kinetics, P450 inhibitory potency, and the type and/or extent of substrate-induced binding spectra using a ferric form of P450 from human liver microsomes. Spectral analysis suggested that the 2-methyl substitution interferes with the stable coordination between the heterocyclic nitrogen of FTIs and P450 heme.

2. Materials and methods

Imidazole-containing FTIs [compounds A–H in Table 1; suffixed numbers (1 and 2) denote imidazole- and 2-methylimidazole-containing FTIs, respectively] were synthesized in the Department of Medicinal Chemistry at Merck Research Laboratories. Pooled human liver microsomes were obtained from the Keystone Skin Bank. Monoclonal anti-CYP3A4 antibody was prepared at Merck Research Laboratories. All other reagents were of analytical grade.

For inhibition studies, the FTI candidate was added at various concentrations to a microsomal reaction mixture (final volume = 250 μL in 0.15 M Tris–HCl buffer, pH 7.4) containing an NADPH-generating system [10 mM glucose-6-phosphate (G6P), 2 IU/mL of G6P dehydrogenase, 10 mM $MgCl_2$], 0.5 mg/mL of protein, 1 mM EDTA, and marker substrate [CYP3A4, 20 μM testosterone; CYP2C9, 10 μM tolbutamide; CYP2D6, 50 μM (±)-bufuralol]. Incubation was carried out at 37° for 20 min, followed by HPLC analysis [17,18]. For the metabolic kinetics study, pooled human liver microsomes (final concentration = 0.5 mg/mL) were incubated with various concentrations of the FTI candidate (0.1 to 20 μM) in an NADPH-fortified reaction mixture (as described above) at 37°. Samples were taken at the designated time points, and the loss of the parent FTI candidate was measured by the LC-MS method described [16]. For the antibody study, microsomes were preincubated with 0–20 μL of monoclonal antibody (ascites)/mg of microsomal protein for 15 min on ice. Metabolism was initiated by the addition of FTI (10 or 50 μM) in an NADPH-fortified reaction mixture (as described above) and continued for 30–90 min at 37°. The remaining substrate was measured by the aforementioned LC-MS method.

Spectral titrations were conducted using a double-beam spectrophotometer (Perkin-Elmer Lambda 20). Microliter volumes of methanol solutions of FTI were added to the experimental cuvette with an equal volume of methanol added to the reference cuvette. Each cuvette contained a 0.5-mL incubation mixture consisting of 0.15 M Tris–HCl buffer (pH 7.4), 1 mM EDTA, and 1 mg/mL of pooled human liver microsomes. After each dilution, the difference spectrum was scanned at 20° from 350 to 500 nm. Preliminary experiments showed that the solvent (methanol) had little effect on the type (and magnitude) of spectra in the concentration range used. The type of substrate-induced binding spectra was determined by the position of the peak (λ_{max}) and the minimum (λ_{min}) wavelengths on the spectrum [19].

3. Results and discussion

Immunoinhibition studies with monoclonal anti-CYP3A4 antibody indicated that the metabolism of the FTI candidates tested in this study was inhibited > 90% by the antibody regardless of the 2-methyl substitution, suggesting that CYP3A4 plays a predominant role in the metabolism of both imidazole- and 2-methylimidazole-containing FTI candidates. The methyl substitution on the imidazole ring dramatically reduced the inhibitory potency of all non-substituted FTI candidates for the marker metabolism catalyzed by CYP3A4 and, to a lesser extent, by CYP2C9 (Table 1). In contrast, this substitution had little effect on the inhibitory potency (IC_{50} ~20 μM) for the CYP2D6-catalyzed metabolism. Non-substituted FTI candidates (**A1**, **B1**, **C1**, and **D1**) all demonstrated concentration-dependent type II binding spectra (max, ~425 nm; min, ~390 nm) with P450,

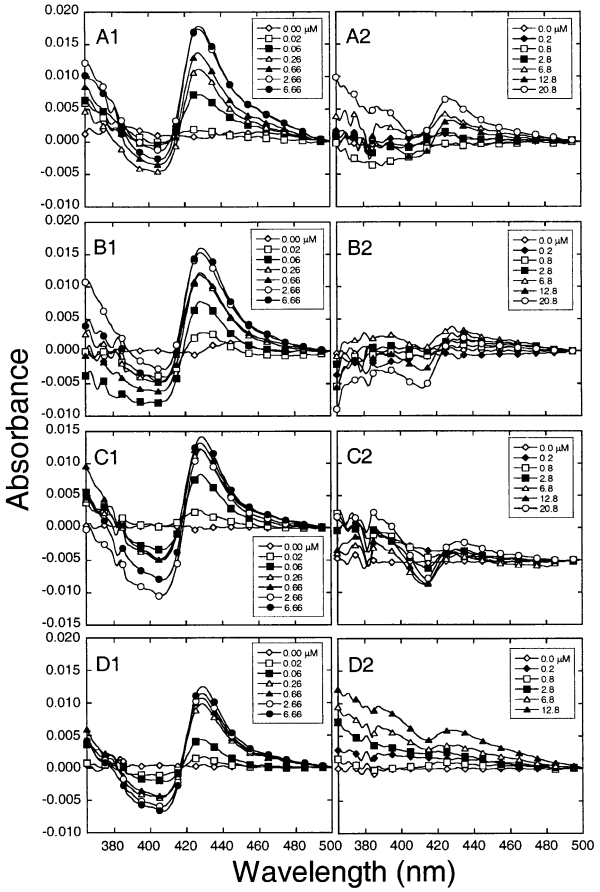


Fig. 1. Substrate-induced P450 binding spectra by imidazole- and 2-methylimidazole-containing FTI candidates in pooled human liver microsomes. Non-substituted imidazole-containing FTIs (**A1**, **B1**, **C1**, and **D1**) showed a typical type II binding spectrum with maximum Soret absorption around 425 nm, while 2-methylimidazole-containing FTIs (**A2**, **B2**, **C2**, and **D2**) showed either very weak type II spectra (**A2**, **B2**, and **D2**) or type I spectrum (**C2**) with minimum Soret absorption around 415 nm.

presumably CYP3A4 (Fig. 1). Consistent with the difference in the inhibitory potency for CYP3A4, the dissociation constants obtained from the spectral titration studies (K_s) for the non-substituted FTIs were much smaller than those for the corresponding 2-methyl-substituted FTIs (Table 2). The 2-methyl substitution markedly weakened the type II binding spectra (**A1**→**A2**, **B1**→**B2**, and **D1**→**D2**) or reversed the type of interaction from type II to type I (**C1**→**C2**). Metabolic kinetics was also examined for non-substituted/2-methyl-substituted analogs (**A1/A2**, **B1/B2**, and **C1/C2**) in human liver microsomes (Table 2). Methyl substitution affected both the K_m and V_{max} values to the same extent. Methyl-substituted **A2**, **B2**, and **C2** had ~12-, ~18-, and ~40-fold larger K_m and V_{max} values than the corresponding non-substituted analogs (**A1**, **B1**, and **C1**, respectively). Thus, the substitution had little effect on intrinsic metabolic clearance (V_{max}/K_m).

Compounds that can bind simultaneously to both the lipophilic region of the P450 protein and to the prosthetic heme iron are known to be inherently more potent inhibitors than those depending on only one of these binding interactions [20]. The binding of inhibitors that are strong iron ligands gives rise to a type II difference spectrum, as seen with the non-substituted imidazole-containing FTIs (Fig. 1). Strong ligands (such as the heterocyclic nitrogen) displace weak ligands (water) from the hexacoordinated heme of P450. This leads to the subsequent coordination to the pentacoordinated heme, resulting in the P450 shift from its high-spin to low-spin dominant form. This spin state change is accompanied by an increase in the redox potential of the P450, which makes P450 reduction (by NADPH P450 reductase) more difficult. Therefore, both the change in redox potential and the physical occupation of the sixth coordination site for oxygen by the strong iron ligand (heterocyclic nitrogen) on the imidazole ring of FTI candidates may be responsible for the potent CYP3A4 inhibition and low turnover rates (V_{max}) with non-substituted imidazole-containing FTIs. One methyl substitution at the 2-position on the imidazole ring dramatically increased V_{max} as well as K_m values (Table 2). This appears to further support the hypothesis that the steric factors play an important role in the interaction between the substrate and active oxygen at the

Table 2
P450 interaction and metabolic kinetics of imidazole- and 2-methylimidazole-containing FTIs in human liver microsomes

Compound	P450 binding		CYP3A4 inhibition (IC_{50}, μM)[a]	Metabolic kinetics		
	Type	K_S (μM)		K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (mL/min/mg)
A1	II	0.0845	0.10	0.256	0.0916	0.358
A2	II?	4.03	8.09	3.22	0.943	0.293
(ratio)		(48)	(81)	(13)	(10)	(0.82)
B1	II	0.0451	0.25	0.144	0.0757	0.526
B2	II?	3.79	9.64	2.87	1.20	0.418
(ratio)		(84)	(39)	(20)	(16)	(0.80)
C1	II	0.0675	0.24	0.169	0.0430	0.254
C2	I	10.5	41.8	7.76	1.46	0.188
(ratio)		(156)	(174)	(46)	(34)	(0.74)

[a] Data are taken from Table 1 for the sake of comparison.

CYP3A4 heme and affect the reactivity (turnover) in CYP3A4-catalyzed metabolism [21].

In summary, the present study in human liver microsomes confirmed previous findings in rats [16] and further demonstrated that the manner of P450 (CYP3A4) interaction with imidazole-containing FTI candidates, probed by the substrate-induced binding spectra, plays an important role in simultaneously determining their turnover (V_{max}) and CYP3A4 inhibitory potency (K_m and K_i). One methyl substitution on the imidazole ring at the 2-position dramatically affected their metabolic profile, due likely to the steric effect on the interaction between the substrate and the P450 (CYP3A4) active site. The substitution on the imidazole ring successfully improved the metabolism profiles of FTI candidates by reducing the potential to inhibit CYP3A4-mediated metabolism without affecting *in vitro* metabolic stability (V_{max}/K_m).

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