CYP4F Enzymes are Responsible for the Elimination of Fingolimod (FTY720), a Novel Treatment of Relapsing Multiple Sclerosis

Yi Jin, Markus Zollinger, Hubert Borell, Alfred Zimmerlin, and Christopher J. Patten

Novartis Institutes for BioMedical Research, Drug Metabolism and Pharmacokinetics, Basel, Switzerland (YJ, MZ, HB, AZ) and BD Biosciences, 6 Henshaw Street, Woburn, MA 01801 (CJP)
Running Title:
CYP4F catalyzes fingolimod hydroxylation

Corresponding Author:
Dr. Yi Jin
Novartis Pharma AG, Fabrikstrasse 14, WSJ-153.2.02, P.O. box, CH-4002 Basel, Switzerland
Telephone: +41 79 535 9601
Fax: +41 61 696 8583
Email: yi.jin@novartis.com

Text pages: 35
Tables: 2
Figures: 7
References: 36
Abstract word count: 244
Introduction word count: 638
Discussion word count: 1495
Abbreviations

CYP, cytochrome P450; DETC, sodium diethyldithiocarbamate; FMO, flavin-containing monooxygenase; 20-HETE, 20-hydroxyeicosatetraenoic acid; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; HPLC-RA, high-performance liquid chromatography with radiodetection; IC_{50}, inhibitor concentration producing 50% inhibition; K_{i}, inhibition constant; K_{m}, Michaelis-Menten affinity constant; LC-MS, liquid chromatography-mass spectrometry; LTB4, leukotriene B4; TAO, troleandomycin; V_{max}, maximum velocity
Abstract

Fingolimod (FTY720, Gilenya™) is a novel drug recently approved in the US for the oral treatment of relapsing multiple sclerosis. The compound is eliminated predominantly by ω-hydroxylation, followed by further oxidation. The ω-hydroxylation was the major metabolic pathway in human liver microsomes (HLM). The enzyme kinetics in HLM were characterized by a Michaelis-Menten affinity constant (K_m) of 183 µM and a maximum velocity (V_max) of 1847 pmol/min/mg. Rates of fingolimod metabolism by a panel of HLM from individual donors showed no correlation with marker activities of any of the major drug metabolizing cytochrome P450 (CYP) enzymes or of flavin-containing monooxygenase (FMO). Among 21 recombinant human CYP enzymes and FMO3, only CYP4F2 (and to some extent CYP4F3B) produced metabolite profiles similar to those in HLM. Ketoconazole, known to inhibit not only CYP3A but also CYP4F2, was an inhibitor of fingolimod metabolism in HLM with an inhibition constant (K_i) of 0.74 µM (and by recombinant CYP4F2 with IC_50 of 1.6 µM), while there was only a slight inhibition found with azamulin and none with troleandomycin. An antibody against CYP4F2 was able to inhibit almost completely the metabolism of fingolimod in HLM whereas antibodies specific to CYP2D6, CYP2E1, and CYP3A4 did not show significant inhibition. Combining the results of these four enzyme phenotyping approaches, we demonstrated that CYP4F2, and possibly other enzymes of the CYP4F subfamily (e.g., CYP4F3B), are the major enzymes responsible for the ω-hydroxylation of fingolimod, the main elimination pathway of the drug in vivo.
**Introduction**

Fingolimod (FTY720, Gilenya™) is recently approved in the US as a new oral drug in the indication relapsing multiple sclerosis. Treatment with fingolimod results in retention of lymphocytes in the lymph nodes, away from the central nervous system where they are involved in inflammation and tissue damage (Kappos et al., 2006). The compound undergoes reversible biotransformation to the active principle fingolimod phosphate (Billich et al., 2003; Albert et al., 2005; Zollinger et al., 2010). The phosphate acts as a potent agonist at sphingosine 1-phosphate receptors on lymphocytes, inducing receptor internalization, hence depriving the lymphocytes from sensing the stimulus to egress from lymphoid tissue (Brinkmann, 2007). Fingolimod itself shows low or negligible activity at these receptors (Hale et al., 2004; Albert et al., 2005).

Fingolimod is cleared slowly with a terminal half-life of approximately 5-6 days in humans (Kovarik et al., 2007, 2009). Even though fingolimod is metabolized by several pathways, including reversible phosphorylation, oxidation, and conjugation with endogenous fatty acids to form ceramide analogs, its elimination occurs predominantly by oxidation, as detailed in an accompanying publication (Zollinger et al., 2010). The oxidative pathway involves ω-hydroxylation at the methyl terminus of the octyl chain, followed by further oxidation to a carboxylic acid and subsequent loss of two carbon units by β-oxidation. The work presented here revealed that the CYP4F subfamily plays a major role in the metabolism of fingolimod.

Of the numerous cytochrome P450 (CYP) enzymes identified in the human genome (Jiang et al., 2010), an increasing number is becoming recognized as clinically relevant (Clarke and Jones, 2008). The CYP1, CYP2, and CYP3 enzyme families are well known to be involved in drug metabolism, while CYP4 enzymes are known mainly for their role in the metabolism of endogenous fatty acids, prostaglandins, and steroids. The human CYP4F subfamily includes CYP4F2, CYP4F3A, CYP4F3B, CYP4F6, CYP4F8, CYP4F11, CYP4F12, and CYP4F22.
(Nelson et al., 2004; Kalsotra and Strobel, 2006; Wang et al., 2009; Jiang et al., 2010). CYP4F2 is expressed in the human liver, intestine, and kidney and catalyzes the ω-hydroxylation of various eicosanoids and related compounds, including arachidonic acid to form 20-hydroxyeicosatetraenoic acid (20-HETE) (Powell et al., 1998; Lasker et al., 2000; Kalsotra and Strobel, 2006). CYP4F3B is very similar in amino acid sequence to CYP4F2 and displays overlapping catalytic activity. Like CYP4F2, CYP4F3B efficiently oxidizes arachidonic acid (Christmas et al., 2001), and both enzymes contribute to the ω-hydroxylation of very long-chain saturated fatty acids in human liver microsomes (HLM) (Sanders et al., 2006). An alternative transcript of the CYP4F3 gene, CYP4F3A, arises from differential promoter use, leading to the incorporation of an alternative exon that changes substrate preferences to favor the ω-hydroxylation of leukotriene (Christmas et al., 1999). In contrast to CYP4F3B, which is selectively expressed in the liver, kidney, trachea, and gastrointestinal tract (Christmas et al., 2001), CYP4F3A is a non-hepatic enzyme expressed in myeloid cells in peripheral blood and bone marrow (Christmas et al., 2001), and in human polymorphonuclear leukocytes (Kikuta et al., 1993).

CYP4F enzymes, including CYP4F2 and CYP4F3B, were recently shown to be the major enzymes catalyzing the initial O-demethylation of the antiparasitic prodrug pafuramidine by HLM, and to catalyze the same reaction in human intestinal microsomes (Wang et al., 2006, 2007). CYP4F11 was found to be able to metabolize a number of drugs, including erythromycin, benzphetamine, ethylmorphine, chlorpromazine, and imipramine (Kalsotra et al., 2004). Hashizume et al. (2002) reported the involvement of intestinal CYP4F12, besides CYP2J2, in the conversion of the prodrug ebastine to the active principle carebastine. Thus, the primal view that CYP4F enzymes are unimportant in drug metabolism is now gradually revised (Kalsotra and Strobel, 2006).
The objective of the current *in vitro* study was to identify the enzymes involved in the hydroxylation of fingolimod. This will provide a basis for assessing potential effects of co-medications and genetic variants on the pharmacokinetics of fingolimod.
Methods

**Study Drug.** [\(^{14}\text{C}\)]Fingolimod hydrochloride ([2-\(^{14}\text{C}\)]-2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) with a radiochemical purity of 98% was synthesized by the Isotope Laboratory of Novartis (Basel, Switzerland). The structure of the compound and the labeling position are shown in Fig. 1.

**Reagents and Chemicals.** Taxol (paclitaxel), quinidine, sulfaphenazole, sodium diethyldithiocarbamate (DETC), and tranylcypromine were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Furafylline was obtained from Ultrafine Chemicals (Manchester, UK). Ketoconazole and troleandomycin (TAO) were purchased from Janssen Chemica (Beerse, Belgium) and azamulin from BD Biosciences (Woburn, MA). NADP disodium salt was from Boehringer (Mannheim, Germany). NADPH, DL-isocitrate, and isocitrate dehydrogenase were from Sigma-Aldrich Chemical. The following chemicals were used to prepare the high-performance liquid chromatography (HPLC) eluents: formic acid (analytical grade, Merck, Darmstadt, Germany), acetonitrile (HPLC grade, Merck), methanol (HPLC grade, Merck), and water (HPLC grade, Fluka, Buchs, Switzerland). Other reagents, chemicals, and buffer salts were from Merck or Fluka and were of analytical or HPLC grade. The reference compound for metabolite M12 was obtained from Yoshitomi Pharmaceutical Industries Ltd. (Tokyo, Japan).

**Human Liver Microsomes, Recombinant Enzymes, and Inhibitory Antibodies.** Pools of HLM and liver microsomes from 12 individual donors were obtained from BD Biosciences (Woburn, MA, USA). The microsomes were characterized by the vendor with regard to the levels of enzyme-selective marker activities (CYP1A2, phenacetin \(O\)-deethylation; CYP2A6, coumarin 7-hydroxylation; CYP2B6, \((S)\)-mephenytoin \(N\)-demethylation; CYP2C8, paclitaxel 6\(\alpha\)-hydroxylation; CYP2C9, diclofenac 4\'\'-hydroxylation; CYP2C19, \((S)\)-mephenytoin 4\'\'-
hydroxylation; CYP2D6, bufuralol 1'-hydroxylation; CYP2E1, chlorzoxazone 6-hydroxylation; CYP3A4, testosterone 6β-hydroxylation; CYP4A11, lauric acid 12-hydroxylation; flavin-containing monooxygenase (FMO), methyl p-tolyl sulfide oxidation). Microsomes prepared from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing recombinant human CYP enzymes or FMO3 and control insect cell membrane preparations were supplied by BD Biosciences. Monoclonal antibodies inhibitory to CYP2D6, CYP2E1, and CYP3A4 were obtained from BD Biosciences. A polyclonal antibody against CYP4F2 was purchased from Fitzgerald Industries International, Inc. (Concord, MA, USA). The antiserum was raised in rabbit using purified human CYP4F2 as immunogen and supplied as lyophilized IgG fraction. The specificity of the polyclonal antibody was determined by Western blotting. The antibody reacted mainly with its corresponding 53 kDa immunogen in HLM. The anti-CYP4F2 was able to inhibit the ω-hydroxylation of oleic acid, arachidonic acid, and LTB4 (all data from the vendor) almost completely. Because of extensive structural homology between CYP4F2 and other CYP4F isoenzymes, reaction of anti-CYP4F2 with other CYP4F isoenzymes could not be excluded. The purity of the antibody was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

**Incubations with HLM and Recombinant Enzymes.** Incubations with HLM and recombinant enzymes (CYP and FMO3) were performed in 25 mM sodium citrate buffer (pH 7.5) at 37°C. After adding different volumes of [14C]fingolimod stock solution, the reactions were started by adding a mixture consisting of 4 µl of a fresh 10 mM NADPH solution and 20 µl of an NADPH-regenerating system containing isocitrate-dehydrogenase (10 U/ml), NADP (10 mM), isocitrate (50 mM), and MgCl₂ (50 mM). The final incubation volume was between 0.2 and 0.4 ml. For establishing linear reaction conditions, different concentrations of microsomal protein
and different incubation times were tested. For analysis of enzyme kinetics, incubations were performed for 15 min using 0.6 mg microsomal protein/ml and 13 different substrate concentrations ranging from 1 to 500 µM. Incubations with recombinant CYP enzymes contained 150 pmol CYP/ml. Incubations with recombinant FMO3 contained 0.4 mg protein/ml. CYP4F2 enzyme kinetic experiments were performed with 100 pmol CYP/ml for 15 min at 37°C using 9 substrate concentrations ranging from 0 to 250 µM. Reactions were terminated by adding between 0.2 and 0.4 ml of ice-cooled ethanol containing 0.5% formic acid (v/v), followed by centrifugation at 30 000 x g for 15 min to remove protein. For the kinetic experiments, aliquots of the supernatants were analyzed by high-performance liquid chromatography with radiodetection (HPLC-RA) using the HPLC conditions 2 (short gradient; see below). For other investigations the supernatants were analyzed by HPLC-RA using HPLC conditions 1 (longer gradient).

**Correlation Analysis.** The rate of fingolimod metabolism was determined in incubations with HLM from 12 individual donors. The incubation time was 24 min, and the protein concentration was 0.7 mg/ml.

**Incubations with HLM in the Presence of Chemical Inhibitors.** Biotransformation of 1 and 50 µM [14C]fingolimod by HLM (0.4 mg protein/ml) was investigated in the presence of different chemical inhibitors. Two concentrations of the inhibitors were used. The concentrations were 2 and 10 µM for furafylline (mechanism-based CYP1A2 inhibitor), taxol (CYP2C8), sulfaphenazole (CYP2C9), tranylcypromine (CYP2C19); 1 and 10 µM for quinidine (CYP2D6), ketoconazole (CYP3A and CYP4F2); 5 and 10 µM for azamulin (CYP3A); 2 and 20 µM for TAO (CYP3A4 specific mechanism-based inhibitor); and 5 and 30 µM for DETC (mechanism-based CYP2E1 inhibitor). Mechanism-based inhibitors were preincubated with HLM in the presence of NADPH before reaction start. For the determination of the inhibition constant (K_i) of
ketoconazole, fingolimod was incubated at 8–9 different concentrations (0–220 µM) with HLM (0.6 mg/ml) for 20 min in the presence of 0, 0.4, 1.3, 4, and 10 µM ketoconazole.

**Incubations with HLM in the Presence of CYP-specific Antibodies.** Biotransformation of 50 µM [14C]fingolimod by HLM (0.5 mg protein/ml) was investigated after pre-incubation with specific antibodies inhibitory to CYP enzymes. The effects of monoclonal antibodies inhibitory to CYP3A4, CYP2E1, CYP2D6, and of a polyclonal anti-CYP4F2 antibody were investigated. The pre-incubations of the HLM were performed using different amounts of antibody at 0°C for 15 min. The incubations with fingolimod were started by the addition of NADPH and NADPH-regenerating system. The incubation time was 20 min.

**HPLC with Radiodetection.** HPLC-RA was performed on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) using an Advantage Armor C18 pre-column (20 x 2.1 mm, 5 µm particles) and an Advantage Armor C18 column (150 x 3 mm, 5 µm particles) from Analytical Sales and Services (Pompton Plains, NJ, USA). A flow rate of 0.5 ml/min was used. The pre-column and column were held at 35°C. The solvent system consisted of 0.5% (v/v) aqueous formic acid (mobile phase A) and 0.5% (v/v) formic acid in acetonitrile/methanol (1:1, v/v; mobile phase B). Two different gradients of mobile phase were developed. A longer gradient ("conditions 1": 0 min/5% B, 10 min/5% B, 40 min/80% B, 47 min/100% B, 55 min/100% B) was used for metabolite profiling. A shorter gradient ("conditions 2": 0 min/50% B, 6 min/50% B, 15 min/85% B, 17 min/100% B, 23 min/100% B) was used for enzyme kinetics, inhibition- and other experiments. For on-line radiodetection, a Berthold (Wildbad, Germany) LB 506 C-1 monitor and Rialuma liquid scintillator (Lumac, Groningen, The Netherlands) were used.

**Structural Characterization of Metabolites by Mass Spectrometry.** For structural characterization, metabolites were generated by incubating 20 µM [14C]fingolimod with HLM
and with recombinant human enzymes (CYP2D6, CYP2E1, CYP3A4, CYP4F2, CYP4F3B, and CYP4F12). The enzymatic reactions were stopped by addition of cold acidified ethanol, the proteins were pelleted by centrifugation, and the supernatants were concentrated by partial evaporation before injection for liquid chromatography-mass spectrometry (LC-MS). The LC-MS instrumentation consisted of an 1100 Series Capillary Pump from Agilent Technologies, a Q-Tof 2 mass spectrometer with an electrospray ion source from Micromass (Wythenshawe, UK), and a Berthold LB 507B detector for parallel radioactivity monitoring. Two different chromatographic conditions were used ("conditions 3" and "conditions 4"). HPLC conditions 3 involved a 150 x 2.1 mm C18 Advantage Armor column (5 µm particles; Analytical Sales and Services) and an eluent gradient of 0.5% (v/v) aqueous formic acid versus 0.5% (v/v) formic acid in methanol/acetonitrile 1:1 (v/v). HPLC conditions 4 involved a 150 x 2.1 mm Symmetry C18 column (3.5 µm particles; Waters, Milford MA, USA) and an eluent gradient of 5 mM aqueous ammonium acetate versus acetonitrile. The electrospray interface was operated in the positive ion mode with nitrogen as nebulizer gas (7 bar), desolvation gas (350 l/h), and cone gas (40 l/h). The desolvation temperature was set to 150°C, the source block was heated to 80°C, and the spray capillary was set to 3.0 kV. Fragment ions were generated by up-front collisional activation in the ion source region at elevated cone voltages. The mass resolution was between 7000 and 9000.

**Data Analysis.** Velocities of enzymatic reactions are reported as pmol/min/mg microsomal protein for incubations with HLM and pmol/min/nmol CYP for incubations with recombinant CYP enzymes. Correlation coefficients (R) were obtained by plotting the metabolic rate of fingolimod versus the marker activity for each CYP enzyme using Microsoft Excel. Inhibitor concentrations producing 50% inhibition (IC50) were determined by linear interpolation. Enzyme kinetic parameters Vmax, Km, and Ki for the biotransformation by HLM and recombinant CYP4F2 were calculated using SigmaPlot 8.0, Enzyme Kinetics module version 1.1 (SPSS Science Inc.,
Chicago, IL, USA). $V_{\text{max}}$ and $K_m$ were determined by nonlinear regression using the Michaelis-Menten model. The goodness of fit ($R^2$) was 0.995 and 0.807 for HLM and CYP4F2, respectively. 95% confidence intervals of the kinetic parameters were determined by the software. The intrinsic clearance ($CL_{int}$) was calculated as $V_{\text{max}}/K_m$. 
Results

Metabolite Profiles. The biotransformation of fingolimod by HLM in the presence of NADPH was slow and produced two detectable metabolites in small amounts. The more abundant of the two was M12, the less abundant was M15 (Fig. 2, Table 1). Additional metabolites (M16 to M20) were observed in incubates with some of the recombinant human CYP enzymes (Table 1). No metabolites were detected in incubates with human liver cytosol alone (data not shown).

Metabolite Structures. Metabolites of fingolimod, formed by HLM and recombinant CYP enzymes, were structurally characterized by LC-MS using up-front collisional activation in the ion source region (elevated cone voltage) and exact mass measurements. Metabolite M12 was identified as the product of a hydroxylation at the terminal methyl group of the octyl chain (Fig. 1). The mass spectra of fingolimod and metabolite M12 are shown in Fig. 3. The spectrum of metabolite M12 as well as its retention times under both HPLC conditions used for LC-MS (conditions 3 and 4) matched those of the respective synthetic reference compound (data not shown). Other metabolites for which structural information could be obtained (M15-M20; Fig. 1) were also products of a single hydroxylation. Since the mass spectra of these metabolites (not shown) were very similar to that of M12, it is likely that the metabolites were hydroxylated at the octyl chain as well (Fig. 1). However, the exact sites of hydroxylation could not be determined. Hydroxylation at the aromatic ring seemed unlikely because of prominent collision-induced losses of all three oxygen atoms in the form of water, together with one molecule of ammonia, resulting in the fragment ions at \( m/z \) 253 (unlabeled) and \( m/z \) 255 (\(^{14}\)C-labeled), as observed for M12. Phenols are unlikely to show prominent collision-induced water losses (Ramanathan et al., 2000). Hydroxylation at one of the two carbon atoms between the aromatic ring and the aminopropanediol moiety could not be excluded but seemed unlikely because of the similarity of
the mass spectra of M15-M20 to that of M12. Some of the metabolites co-eluted under the conditions of the radiochromatography (Table 1), but could be distinguished using two different chromatographic systems (conditions 3 and 4) in the LC-MS runs. Under the HPLC conditions 1 (radiochromatography) and 3 (LC-MS analysis), M12 co-eluted with M18 and M15 co-eluted with M16 and M17. Under the HPLC conditions 4 (LC-MS analysis), M12 co-eluted with M17, whereas M15, M16, M18, M19, and M20 eluted as single components. M12 and M17 could be differentiated based on the relative intensity of the fragment ion at \( m/z \) 271/273 ([M+H-2H2O-NH3]+), which was higher in the spectrum of M12 compared with M17.

**Enzyme Kinetics of Fingolimod Metabolism.** After establishing linear reaction conditions regarding incubation time and protein concentration, the dependence of fingolimod metabolism on the substrate concentration was investigated in HLM. Fingolimod, at 13 concentrations ranging from 1 to 500 µM, was incubated with pooled HLM (0.6 mg protein/ml) for 15 min. The plot of velocity (total metabolite formation) versus substrate concentration showed an atypical "bell-shaped" curve (Fig. 4). The decrease of the metabolic rate at high substrate concentrations suggested CYP inhibition or inactivation. The reason for the atypical kinetics was not investigated. An Eadie-Hofstee plot over the 35 to 220 µM concentration range showed a straight line (Fig. 4 inset left), suggesting a single \( K_m \). Non-linear regression analysis of the rate of metabolism versus fingolimod concentration in the range of 35 to 220 µM revealed a \( K_m \) of 183±19 µM (mean±standard error; 95% confidence interval: 134 to 232 µM) and a \( V_{max} \) of 1847±110 pmol/min/mg (mean±standard error; 95% confidence interval: 1565 to 2129 pmol/min/mg). The intrinsic clearance in HLM was low (10 µl/min/mg).

**Correlation Analysis of Fingolimod Metabolism Using a Panel of HLM from Individual Donors.** HLM from 12 individual donors were tested for their ability to metabolize fingolimod. The metabolic rates varied only little between the donors (2–3 fold; data not shown). No
correlation between these rates and any of the enzyme-specific probe substrate activities was found. All correlation values (R) were below 0.5 (Table 2). These results did not provide an indication for an involvement of the enzymes investigated (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5, CYP4A, and FMO) in the metabolism of fingolimod in HLM.

**Biotransformation of Fingolimod by Recombinant Human CYP Enzymes.** Microsomes prepared from baculovirus-infected insect cells expressing single human CYP isoenzymes were used to assess the involvement of these enzymes in the biotransformation of fingolimod. Incubations with a panel of 21 recombinant human CYP enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP3A7, CYP4A11, CYP4F2, CYP4F3A, CYP4F3B, CYP4F12, and CYP19) were conducted under similar conditions for each isoenzyme using 5 and 100 µM fingolimod and 150 pmol CYP/ml. Incubations with FMO3 were performed with 0.4 mg protein/ml. Five recombinant enzymes (CYP2D6, CYP2E1, CYP3A4, CYP4F2, and CYP4F12) showed notable metabolic activity. Low activity was found also with CYP4F3B, while only traces of metabolites or none at all were detected with the rest of the investigated enzymes. The metabolite profiles obtained with selected CYP enzymes and HLM are summarized in Table 1. In addition to the two metabolites observed in HLM incubates (M12 and M15), further hydroxylated metabolites (M16-M20; Fig. 1) were produced by the recombinant CYP2D6, CYP3A4, and CYP4F12.

The metabolite patterns obtained with CYP4F2 and CYP4F3B (Table 1) were most similar to those observed in HLM among all investigated isoenzymes, with M12 being the major metabolite. In addition, small amounts of M15 were detected with CYP4F2, as with HLM. The
rate of metabolism by CYP4F3B was relatively low, representing about one-third to that by CYP4F2. CYP2D6 metabolized fingolimod to at least four hydroxylated metabolites (M12, M15, M16, M18), with M15 as the most abundant metabolite. CYP2E1 produced M15 as the only metabolite of fingolimod. In the incubations with recombinant CYP3A4, six hydroxylated metabolites were characterized by LC–MS (M15–M20) of which M20 was the major one. M15 was a very minor component, accounting for only 3% of the total metabolites formed by CYP3A4. CYP4F12 metabolized fingolimod to M15 and M17 in a 2:3 ratio. Enzyme kinetic parameters for recombinant CYP4F2 were determined from incubations with fingolimod at different substrate concentrations (Fig. 4, inset right). As with HLM, an atypical "bell-shaped" curve was found with a decrease of the metabolic rate at high substrate concentrations. A $K_m$ of 45 $\mu$M and a $V_{\text{max}}$ of 1049 pmol/min/nmol CYP were determined by non-linear regression analysis of the metabolism rate versus fingolimod concentration using the concentration range of 10 to 100 $\mu$M. The intrinsic clearance was 23 $\mu$l/min/nmol CYP.

Inhibition of Fingolimod Metabolism by CYP-specific Chemical Inhibitors. CYP-selective chemical inhibitors were used as the next approach for enzyme phenotyping (Fig. 5). The metabolism of fingolimod in HLM was most strongly inhibited by ketoconazole (1 and 10 $\mu$M), a known CYP3A4 inhibitor. The inhibition was almost complete using 10 $\mu$M ketoconazole with 1 $\mu$M substrate concentration. However, there was no inhibition by TAO (2 and 20 $\mu$M), a mechanism-based inhibitor for CYP3A4. Azamulin (5 and 10 $\mu$M), a potent and irreversible CYP3A inhibitor (Stresser et al., 2004), showed only slight inhibition (13–27%) of fingolimod metabolism in HLM. No significant inhibition of fingolimod metabolism was observed with furafylline, taxol, sulphaphenazole, tranylcyromine, quinidine, and DETC (Fig. 5).
Since no relevant role of CYP3A4 was found in the experiments with recombinant CYP enzymes (Table 1) and the strongest inhibition was observed with ketoconazole, which is known to inhibit CYP4F2 and CYP4F12 (Stresser et al., 2004), besides CYP3A4, the effects of ketoconazole on fingolimod metabolism by recombinant CYP4F2 and CYP4F12 were investigated. The biotransformation of 10 µM fingolimod by 100 pmol/ml CYP4F2 and 50 pmol/ml CYP4F12 was readily inhibited by ketoconazole with IC₅₀ values of 1.6 and 0.6 µM, respectively.

The inhibition of the metabolism of fingolimod in HLM by ketoconazole was further investigated at different substrate concentrations in the presence (0.4–10 µM) or absence of ketoconazole under linear conditions with regard to incubation time and protein concentration (Fig. 6). The rates of biotransformation were analyzed by nonlinear curve-fitting using the Enzyme Kinetics module of SigmaPlot, considering different inhibition types: full or partial competitive, noncompetitive, uncompetitive, and mixed inhibition. The models were ranked by the goodness of fit (Akaike’s information criterion). The partial competitive inhibition model provided the best fit of the data with an inhibition constant Kᵢ of 0.74 ± 0.09 µM.

**Inhibition of Fingolimod Metabolism in HLM by CYP-specific Inhibitory Antibodies.**

Fig. 7 shows the effects of CYP-specific inhibitory antibodies on the metabolism of 50 µM fingolimod by HLM (0.5 mg/ml). There were only small changes to the rate of metabolism, or none at all, in the presence of the monoclonal antibodies inhibitory to CYP2D6, CYP2E1, and CYP3A4. In contrast, polyclonal antibodies raised against human liver CYP4F2 were found to be very potent inhibitors of fingolimod metabolism in HLM. The maximum inhibition of total metabolic activity was 93%. In a parallel experiment with recombinant CYP4F2, anti-CYP4F2 showed a similar maximum inhibition (92%) of fingolimod metabolism.
Discussion

The \textit{in vitro} metabolism of fingolimod was investigated in HLM and recombinant human CYPs to determine the enzymes involved in the oxidative metabolism of the compound. The biotransformation of fingolimod in HLM was slow with an intrinsic clearance of 10 µl/min/mg microsomal protein. This is consistent with the low clearance of the drug found after intravenous administration (6.3 l/h; Kovarik et al., 2007). In humans \textit{in vivo}, fingolimod was found to be metabolized by several pathways, including reversible phosphorylation to the pharmacologically active principle. However, the elimination of the compound occurred predominantly by oxidation. The initial step of the oxidative pathway was hydroxylation at the terminal methyl group of the octyl chain (metabolite M12). Because of rapid further oxidation to a carboxylic acid and subsequent $\beta$-oxidation, M12 was not detectable \textit{in vivo}. Yet, in HLM, lacking the enzymes catalyzing the further oxidation of the primary hydroxylation product, M12 was the major biotransformation product. In addition, metabolite M15 was observed in HLM as a minor hydroxylation product, and other hydroxylated metabolites (M16–M20) were found in incubates with recombinant CYP enzymes. However, M15 to M20 appeared to play no relevant role because neither these metabolites nor any downstream products could be identified \textit{in vivo} (Zollinger et al., 2010).

Among the six recombinant CYP enzymes that showed notable metabolism of fingolimod, CYP4F2 and CYP4F3B produced metabolite profiles most similar to those produced by HLM. M12 was the major metabolite formed by CYP4F2 and CYP4F3B (Table 1). The rate of metabolism by CYP4F3B was three times lower, compared with CYP4F2. In contrast to CYP4F2 and CYP4F3B, CYP2D6, CYP2E1, CYP3A4, and CYP4F12 showed metabolite profiles different from those in HLM. The main metabolites produced by these enzymes (Table 1), all being isomers of M12, were not observed \textit{in vivo}. This might be due to differences between
recombinant and natural enzymes (Proctor et al., 2004) in their catalytic competency (Ogilvie et al., 2008). Due to the “artificial” nature of expressed enzymes, differences in activity characteristics may exist with respect to P450s in their native membrane environment (Bjornsson et al., 2003). Therefore, CYP2D6, CYP2E1, CYP3A4, and CYP4F12 did not seem to contribute significantly to the metabolism of fingolimod \textit{in vivo}. Other CYP enzymes and recombinant FMO3 generated only traces of fingolimod metabolites and are therefore unlikely to contribute as well.

Abundance values of CYP4F2 in HLM of 142 pmol/mg (Jin et al., 1998) and 155 pmol/mg (Lasker et al., 2000) have been reported previously. A new method using anti-CYP4F2 peptide IgG showed highly variable CYP4F2 contents in HLM with levels ranging between 0 and 80 pmol/mg (Hirani et al., 2008). A recent human liver proteome analysis (Jiang et al., 2010) revealed high protein abundance of CYP4F2 and CYP4F3, representing 42% and 29%, respectively, of the CYP3A4 abundance. Lasker et al. (personal communication) measured the hepatic CYP4F contents in 29 donors and obtained values ranging from 18 to 128 pmol/mg microsomal protein. According to these results, the hepatic abundances of CYP4F are relatively high. The liver content of CYP4F12 is currently unknown (Jiang et al., 2010). While mRNA levels of CYP4F2, CYP4F3B, and CYP4F11 were measurable in the liver, the transcript of \textit{CYP4F12} was barely expressed, according to a relative abundance study (Lasker, personal communication). In view of the results with recombinant enzymes, CYP4F2, and to some extent CYP4F3B, appear to be the major catalysts of fingolimod hydroxylation.

In a panel of individual HLM, no correlation of fingolimod metabolism with CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4/5, CYP4A,
and FMO marker activities was found. This is in line with CYP4F being the major fingolimod metabolizer.

In agreement with the above results, no significant inhibition of fingolimod metabolism by the chemical CYP-selective inhibitors furafylline, taxol, sulfaphenazole, tranylcypromine, quinidine, DETC, and TAO were found. Surprisingly, a potent in vitro inhibition by the CYP3A4 inhibitor ketoconazole was observed, in contrast to the absence of an inhibitory effect of TAO, a mechanism-based inhibitor for CYP3A4. However, ketoconazole had also been shown previously to inhibit CYP4F2 (Stresser et al., 2004) although at higher concentrations than for CYP3A4. In line with these results, ketoconazole also inhibited the metabolism of fingolimod by recombinant CYP4F2 (IC$_{50}$ = 1.6 µM). Wang et al. (2006) found a strong selectivity of ketoconazole for CYP4F2 versus CYP4F3B and proposed this inhibitor as a tool to distinguish the two closely related enzymes. Therefore, the strong effect of ketoconazole on the metabolism of fingolimod in HLM (almost 100% inhibition at 10 µM ketoconazole and 1 µM fingolimod; Fig. 5) suggests a predominant contribution of CYP4F2 and little involvement of CYP4F3B. Azamulin, a potent and irreversible CYP3A inhibitor, showed only moderate inhibition (by 13–27%) of fingolimod metabolism in HLM at concentrations of 5 and 10 µM where CYP3A activity is strongly inhibited. This is consistent with the results of Stresser et al. (2004) who found that azamulin also inhibits CYP4F2 (IC$_{50}$ = 46 µM). Hence, the effect observed with azamulin was likely caused by a partial inhibition of CYP4F2. Together, the data from chemical inhibitors support the major role of CYP4F2 and little involvement of other CYP enzymes in the hepatic metabolism of fingolimod.

A final confirmation of the major contribution of CYP4F enzymes in fingolimod metabolism was obtained from investigations using CYP-specific antibodies. While anti-CYP2D6, anti-
CYP2E1, and anti-CYP3A4 showed no or little effect at concentrations which strongly inhibited the metabolism of their known specific substrates (results not shown), an antibody against CYP4F2 was found to be a very potent inhibitor of fingolimod metabolism by HLM, producing a maximum inhibition of 93%.

Taken together, four approaches were used in the present investigation to identify the major oxidizing enzymes involved in the biotransformation of fingolimod. Correlation analysis using a panel of HLM from individual donors revealed no correlation of fingolimod metabolism with any of the investigated human CYP and FMO enzymes (which did not include CYP4F). Recombinant CYP4F2 showed the highest activity in metabolizing fingolimod to M12, the main metabolite in HLM. Experiments with selective chemical inhibitors supported the major role of CYP4F2. The antibody against CYP4F2 inhibited almost completely the metabolism of fingolimod in HLM. All the four enzyme phenotyping approaches (Ogilvie et al., 2008) were in line with the conclusion that CYP4F enzymes are the major contributors to the hydroxylation of fingolimod. On the basis of our data, CYP4F2 seems to play a major role. However, contributions by other members of the CYP4F subfamily, including CYP4F3B, for which only fragmentary data and experimental tools are available at this time, cannot be excluded. The high preference of CYP4 enzymes for $\omega$- over ($\omega$-1)-hydroxylation of fatty acid chains (Ortiz de Montellano, 2008) is well in line with our results.

Ketoconazole inhibited the biotransformation of fingolimod by HLM with an inhibition constant $K_i$ of 0.74 µM. This value was in line with the IC$_{50}$ value determined with recombinant CYP4F2 (1.6 µM). The in vitro data suggest that ketoconazole may have the potential to inhibit the metabolic clearance of fingolimod. Therapeutic plasma concentrations of ketoconazole are in the range of 2–12 µM (Schulz and Schmoldt, 2003). These values are 3- to 16-fold above the $K_i$ value. Therefore, ketoconazole has a potential to inhibit the metabolic clearance of fingolimod.
In a clinical study, ketoconazole was used as a putative CYP4F2 perpetrator to quantify its influence on fingolimod pharmacokinetics in healthy subjects. A modest average 1.7-fold increase of fingolimod exposure (area under the blood concentration time curve) was found (Kovarik et al., 2009). The \textit{in vivo} data confirmed the clinical relevance of CYP4F2 inhibition by ketoconazole. A \( K_i \) value of ketoconazole on CYP4F2 has not been published. The value determined in this work may find applications for predicting effects of ketoconazole on metabolism of CYP4F2 substrates with the \( I/K_i \) approach (Bjornssen et al., 2003) or using modeling and simulation tools such as DDI Predict\textsuperscript{®} and Simcyp\textsuperscript{®} (Rostami-Hodjegan and Tucker, 2007).

A recent genome-wide association study revealed CYP4F2 as one of three principal genetic determinants of warfarin dose, the other two being CYP2C9 and vitamin K epoxide reductase complex, subunit 1 (Takeuchi et al., 2009). Ward et al. (2008) demonstrated a significant association of the CYP4F2 GA/AA genotype with elevated systolic blood pressure and significantly increased urinary 20-HETE excretion. Hence, genetic polymorphism of CYP4F2 might possibly contribute to the interindividual variability in the metabolism of fingolimod. However, functional consequences of CYP4F2 polymorphism were found to be substrate dependent (Stec et al., 2007). The amino acid change at position 433 (V433M) decreased 20-HETE production by 34–44\% but had no effect on \( \omega \)-hydroxylation of LTB4. A preliminary pharmacogenetic analysis indicated no significant effect of CYP4F2 genetic polymorphism on the pharmacokinetics of fingolimod (unpublished Novartis internal data).

In conclusion, CYP4F enzymes, in particular CYP4F2, were identified as the major catalysts of fingolimod oxidation. The lack of involvement of the common drug-metabolizing enzymes in
fingolimod clearance suggests a reduced drug interaction potential. This provides an advantage regarding the safety of this novel oral therapy for relapsing multiple sclerosis.
Acknowledgements. The authors would like to thank Dr. Rhys Salter (Isotope Lab, Novartis) for the synthesis of the radiolabeled drug, and Mrs. Claudia Sayer for her technical support in the LC–MS analysis. They also thank Dr. Jerome Lasker (Puracyp Inc. Carlsbad, CA) for helpful discussion and for providing unpublished information on CYP4F enzymes, Drs. Shivani Mittra and Rama Sivasubramanian (IISO, Novartis Healthcare Pvt. Ltd.) for assistance in preparing the manuscript, and Drs. Gian Camenisch, Heike Gutmann, Pieter J. Swart, and Olivier Kretz for critical review of the paper.
Authorship Contributions.

Participated in research design: Jin, Zimmerlin, and Patten.

Conducted experiments: Jin, Zollinger, Borell, Zimmerlin, and Patten.

Performed data analysis: Jin, Zollinger, Borell, Zimmerlin, and Patten.

Wrote or contributed to the writing of the manuscript: Jin, Zollinger, Zimmerlin, and Patten.
References


Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, and Nebert DW (2004) Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including
nomenclature recommendations for genes, pseudogenes and alternative-splice variants.

*Pharmacogenetics* **14**: 1-18.


Footnotes.

**Address for reprint requests:** Dr. Yi Jin, Novartis Pharma AG, Fabrikstrasse 14, WSJ-153.2.02, P.O. box, CH-4002 Basel, Switzerland. E-mail: yi.jin@novartis.com
Legends for figures.

Fig. 1. Structures of fingolimod, metabolite M12 (confirmed by comparison with synthetic reference compound), and metabolites M15-M20 (proposed structures based on mass spectral information).

Fig. 2. Radiochromatograms of 30 min incubates of fingolimod with HLM (20 µM substrate, 0.56 mg microsomal protein/ml) and CYP4F2 (100 µM substrate, 150 pmol CYP/mL).

Fig. 3. Electrospray mass spectra of 14C-labeled fingolimod (A) and 14C-labeled metabolite M12 (B); degree of 14C-labeling ~45%. Fragments generated by up-front collisional activation in the ion source region (60 V cone voltage).

Fig. 4. Enzyme kinetic analysis of fingolimod metabolism by HLM. The reaction mixture contained 0.6 mg/mg HLM and 35–500 µM [14C]fingolimod. Reactions were carried out for 15 min. Symbols denote mean ± S.D. of triplicate incubations. Eadie-Hofstee plot (left upper inset) was obtained for substrate concentrations between 35 and 220 µM. The right upper inset shows the enzyme kinetic plot for recombinant CYP4F2 (100 pmol/mL) obtained with 15 min incubation time and 10–200 µM [14C]fingolimod.

Fig. 5. Effect of CYP isoenzyme-selective chemical inhibitors on fingolimod metabolism in HLM. [14C]Fingolimod was incubated with HLM (0.4 mg/ml) in the presence of different inhibitors, and the rates of total metabolite formation (M12 plus M15) were determined. Reactions were carried out for 20 min. The data are shown as mean and range of duplicate determinations. The concentration of fingolimod was 1 µM (white bars) and 50 µM (black bars) for all assays with the exception of azamulin inhibition where the fingolimod concentration was 30 µM (white bars) and 60 µM (black bars). Controls (100% activity) were run with inhibitor vehicle (methanol).
Fig. 6. Inhibition of fingolimod metabolism by ketoconazole (Kᵢ determination). The concentration-dependent kinetics of the metabolism of [¹⁴C]fingolimod (60–220 µM) by HLM (0.6 mg/ml) were determined in the presence of five different concentrations of the inhibitor (I = 0, 0.4, 1.3, 4, 10 µM). The incubation times were 20 min. Data are shown as Michaelis-Menten plot (mean ± S.D. of at least 2 replicates). The inhibition type was found to be partial competitive and the Kᵢ value was determined to be 0.74 µM.

Fig. 7. Effect of CYP-specific inhibitory antibodies on the metabolism of fingolimod by HLM. The total biotransformation rate (formation of M12 plus M15) of fingolimod (50 µM) by HLM (0.5 mg/ml) was investigated after pre-incubation with antibodies inhibitory to CYP2D6, CYP2E1, CYP3A4, and CYP4F2. The results are expressed as activities relative to the control rates in the absence of antibodies.
Table 1

Chromatographic characteristics and relative abundance of structurally characterized fingolimod metabolites in incubates with HLM and microsomes expressing specific human CYP enzymes

<table>
<thead>
<tr>
<th></th>
<th>M12</th>
<th>M15</th>
<th>M16</th>
<th>M17</th>
<th>M18</th>
<th>M19</th>
<th>M20</th>
<th>Fingolimod</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC conditions 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.7</td>
<td>34.3</td>
<td>34.3</td>
<td>34.3</td>
<td>34.6</td>
<td>35.8</td>
<td>36.2</td>
<td>41.9</td>
</tr>
<tr>
<td>HPLC conditions 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.7</td>
<td>35.4</td>
<td>35.4</td>
<td>35.4</td>
<td>35.7</td>
<td>36.8</td>
<td>37.2</td>
<td>42.7</td>
</tr>
<tr>
<td>HPLC conditions 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.1</td>
<td>31.8</td>
<td>32.3</td>
<td>32.1</td>
<td>32.9</td>
<td>34.3</td>
<td>34.8</td>
<td>42.4</td>
</tr>
</tbody>
</table>

Relative abundance of metabolites (%)<sup>c</sup>

<table>
<thead>
<tr>
<th></th>
<th>M12</th>
<th>M15</th>
<th>M16</th>
<th>M17</th>
<th>M18</th>
<th>M19</th>
<th>M20</th>
<th>Fingolimod</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>1.40</td>
<td>0.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>1.02</td>
<td>1.88</td>
<td>1.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td></td>
<td>2.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.11</td>
<td>0.19</td>
<td>0.19</td>
<td>0.38</td>
<td>0.22</td>
<td></td>
<td></td>
<td>1.09</td>
</tr>
<tr>
<td>CYP4F2</td>
<td>2.99</td>
<td>0.31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP4F3B</td>
<td>0.84</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP4F12</td>
<td></td>
<td>1.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.79</td>
</tr>
</tbody>
</table>

Control (insect cell)<sup>d</sup>

<sup>a</sup>: Conditions of HPLC runs with radioactivity detection (cf. experimental part)

<sup>b</sup>: Conditions of LC-MS runs (cf. experimental part)
Relative abundance: percent of total radioactivity in incubate. Incubates of $[^{14}\text{C}]$fingolimod (20 µM for CYP2D6, CYP2E1, CYP3A4, HLM or 100 µM for CYP4F2, CYP4F3B, CYP4F12, and insect cell control) for 30 min with 0.56 mg protein/ml (HLM), 150 pmol CYP/mL (recombinant enzymes), or 0.75 mg protein/mL (insect cell control).

None of the metabolites M12 or M15 to M20 detected.
Table 2

_Correlation analysis of fingolimod metabolism rates with marker enzyme activities in a panel of HLM from individual donors_

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Marker reaction</th>
<th>Correlation (R value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>0.08</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>0.2</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>(S)-Mephenytoin N-demethylation</td>
<td>0.25</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Paclitaxel 6α-hydroxylation</td>
<td>0.18</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4'-hydroxylation</td>
<td>0.4</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin 4'-hydroxylation</td>
<td>0.03</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Bufuralol 1'-hydroxylation</td>
<td>0.39</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>0.32</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Testosterone 6β-hydroxylation</td>
<td>0.32</td>
</tr>
<tr>
<td>CYP4A</td>
<td>Lauric acid 12-hydroxylation</td>
<td>0.32</td>
</tr>
<tr>
<td>FMO</td>
<td>Methyl p-tolyl sulfide oxidation</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Fig. 1

*: position of $^{14}$C-labeling

Fingolimod

M12

M15, M16, M17, M18, M19, M20
Fig. 2

Human liver microsomes

CYP4F2

Radioactivity vs. Retention time (min)

Radioactivity vs. Retention time (min)
Fig. 4

Eadie-Hofstee plot

HLM

CYP4F2

Velocity (pmol/min/mg)

Fingolimod concentration (μM)

Velocity (pmol/min/mmol)

Fingolimod concentration (μM)

HLM
Fig. 5

The diagram illustrates the relative activity (% of control) for various compounds. The x-axis represents different concentrations and compounds, while the y-axis shows the relative activity. The bars indicate the mean values with error bars representing the standard deviation. The compounds and concentrations included are 2 µM Furafylline, 10 µM Furafylline, 2 µM Taxol, 10 µM Sulfaphenazole, 2 µM Tranylcypromine, 10 µM Tranylcypromine, 10 µM Quinidine, 1 µM Quinidine, and others at varying concentrations.
Fig. 6

![Graph showing velocity (pmol/min/mg) vs. fingolimod concentration (μM). Different symbols represent various fingolimod concentrations: 0 μM (black circle), 0.4 μM (white circle), 1.3 μM (black triangle), 4.0 μM (white triangle), and 10 μM (black square). The data points are accompanied by error bars.]