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CYTOCHROME P450 3A4 IS THE MAJOR ENZYME RESPONSIBLE FOR THE METABOLISM OF LAQUINIMOD, A NOVEL IMMUNOMODULATOR

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Abbreviations: EAE, experimental autoimmune encephalomyelitis;CYP, cytochrome P450;MS, multiple sclerosis; HPLC, high performance liquide chromatography;CL_{Int}, clearance intrinsic

ABSTRACT:

In the present study the involvement of cytochrome P450 enzyme(s) in the primary metabolism of laquinimod, a new orally active immunomodulator, has been investigated in human liver microsomes. Hydroxylated and dealkylated metabolites were formed. The metabolite formation exhibited single enzyme Michaelis-Menten kinetics with apparent K_M in the range of 0.09-1.9 mM and V_{max} from 22 to 120 pmol/mg/min. A strong correlation between the formation rate of metabolites and 6β-hydroxylation of testosterone was obtained within a panel of liver microsomes from 15 individuals (r^2 =0.6-0.94). Moreover, ketoconazole and troleandomycin, specific inhibitors of CYP3A4 metabolism demonstrated a significant inhibition of laquinimod metabolism. Furthermore, in incubations with recombinant CYP3A4 all the primary metabolites were formed. In vitro interaction studies with CYP3A4 substrates and possible concomitant medication demonstrated that laquinimod inhibits the metabolism of ethinyl estradiol with an IC_{50} value of about 150 μM, which is high above the plasma level of laquinimod after clinically relevant doses. Ketoconazole, troleandomycin, erythromycin, prednisolone and ethinyl estradiol inhibited the metabolism of laquinimod and IC_{50} values of 0.2, 11, 24, 87, and 235 μM were calculated, respectively. In conclusion, the present study demonstrates that laquinimod is a low affinity substrate for CYP3A4 in human liver microsomes. The likelihood for *in vivo* effects of laquinimod on the metabolism of other CYP3A4 substrates is minor. However, inhibitory effects on the metabolism of laquinimod by potent and specific inhibitors of CYP3A4 such as ketoconazole, are anticipated and should be considered in the continued clinical program for laquinimod.

Laquinimod (ABR-215062, N-ethyl-N-phenyl-5-chloro-1, 2-dihydroxy-1-methyl-2-oxo-3-quinoline-carboxamide) represents a novel orally active immunomodulator similar in structure to the previously clinically evaluated analogue, roquinimex (Linomide®), (Andersen et al., 1996, Karussis et al., 1996, Noseworthy et al., 2000, Tan et al., 2000, Tuvesson et al., 2000). Both roquinimex and laquinimod have been found to effectively inhibit disease in both acute experimental autoimmune encephalomyelitis (EAE)[1)] and chronic relapsing EAE, two mouse models for the study of multiple sclerosis (MS) (Karussis et al., 1993a, Karussis et al., 1993b, Brunmark et al., 2002, Jönsson et al., 2004). MS is an inflammatory disease of the central nervous system characterised by localised leukocyte inflammation and demyelination resulting in nerve cell dysfunction. The prevalence of this severe autoimmune disease varies greatly mirroring that MS is caused by interplay of environmental and genetic factors. Laquinimod has recently successfully been evaluated in a clinical phase II study, where proof of concept was demonstrated in MS patients (Polman et al., 2004) and is now planned to accomplish a clinical phase III program.

The pharmacokinetic properties of laquinimod have been studied in several preclinical species used for the pharmacological and toxicological evaluation of the compound. In mouse, rat, rabbit and dog laquinimod pharmacokinetics is characterised by a high oral bioavailability, a low total clearance and small volume of distribution (unpublished results). Furthermore, the compound seems to be eliminated by metabolism and both hydroxylated and demethylated products have been detected in urine from all the studied species. Hydroxylation and demethylation are reactions known to be catalysed by the cytochrome P450 (CYP) enzymes (Nelson et al., 1996). This large family of enzymes, bound to the endoplasmic reticulum, catalyse a variety of reactions of xenobiotic metabolism as well as metabolism of endogenous compounds.

In the present study the primary metabolism of laquinimod was investigated using different enzymatic preparations including human liver microsomes and recombinantly expressed enzymes.

In addition, *in vitro* studies were performed to predict possible drug-drug interactions of laquinimod in man.

Materials and Methods

Chemicals. ^{14}C-laquinimod (batch rd-A, with a specific radioactivity of 56.0 mCi/mmol and with a radiochemical purity >95%) and the non-radioactive reference compounds, laquinimod, ABR-215791 (M1), ABR-218287 (M3), ABR-218373 (M4), ABR-215818 (M5), and ABR-215174 (M6) were synthesised at Active Biotech Research AB, Lund, Sweden. ^{3}H-ethinyl estradiol (specific radioactivity of 52.3 Ci/mmol) was purchased from Life Science Products, Inc. Boston, Ma. NADPH, coumarin, diclofenac, quinidine, sulphaphenazole, troleandomycin, erythromycin, ethinyl estradiol, and prednisolone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chlorzoxazone, furafylline and ketoconazole were obtained from Research Biochemicals International (Natick, MA, USA). All other chemicals were of analytical grade.

Enzyme systems. Pooled human liver microsomes and a panel of liver microsomal samples from 15 individual livers (Reaction Phenotyping Kit) were obtained from Xenotech, (Cambridge, Kansas, USA).

The human microsomes had been characterised with respect to total CYP content and specific content of individual CYP enzymes, which were determined from their catalytic activity for the biotransformation of the following substrates: CYP1A2 (7-Ethoxyresorufin O-dealkylation), CYP2A6 (coumarine 7-hydroxylation), CYP2B6 (7-Ethoxy-trifluoromethylcoumarin O-deethylation), CYP2C8 (paclitaxel 6□-hydroxylation), CYP2C9 (tolbutamide methyl-hydroxylation), CYP2C19 (S-mephenytoin 4-hydroxylation), CYP2D6 (dextromethorphan O-

demethylation), CYP2E1 (chlorzoxazone 6-hydroxylation) and CYP3A4/5 (testosterone 6 β-hydroxylation).

cDNA expressed human CYP1A2, 2B6, 2C9, 2C19, 2D6, and 3A4 were obtained from Gentest (Woburn, MA, USA).

Assay conditions. Incubations were performed with laquinimod (5-3000 μM) and 1-4 mg/mL microsomal protein in 50 mM sodium phosphate buffer, pH 7.4 at 37°C for 0-60 min. Preliminary experiments were performed to established conditions for reasonable substrate consumption and time and protein linearity of metabolite formation. The incubation mixture was preincubated for 5 min prior to addition of NADPH (10 mM, final concentration). The reaction was stopped by addition of an equal volume of ice-cold acetone and centrifuged at 10,000xg for 10 min. The supernatant was transferred to autosample vials and analysed by high performance liquid chromatography (HPLC).

For identification of metabolites, incubations were performed with laquinimod (1500 μM) and 2 mg of microsomal protein for 60 min.

In the correlation studies, the metabolism of laquinimod was determined across 15 different human liver microsomal preparations, which had been characterised for specific CYP substrate activities. To give an appropriate evaluation of the metabolism for this correlation study, 300 μM laquinimod was incubated with 3 mg/mL microsomal protein.

In the inhibitions studies, incubations were performed with human liver microsomes and specific inhibitors of CYP enzymes. Laquinimod, 200 μM, was incubated in 3 mg/ml microsomal protein

and one of the following enzyme inhibitors: furafylline, 20 μM (Kunze and Trager, 1993), coumarin, 25 μM (Pearce et al., 1992), diclofenac, 200 μM (Transon et al., 1996), sulfaphenazole, 50 μM (Relling et al., 1990), quinidine, 25 μM (Inaba et al., 1985), chlorzoxazone, 50 μM (Peter et al., 1990), ketoconazole, 2 μM (Baldwin et al., 1995), or troleandomycin, 100 μM (Guengerich, 1990) at pH 7.4 for 20 min at 37°C. Each inhibitor was added in ethanol (final concentration of ethanol in each incubation was 2%). Control experiments with 2% ethanol and control experiments without NADPH were performed in parallel. All samples were made in duplicate. There was a preincubation equilibrium and inactivation period for 10 min at 37°C, with inhibitor and NADPH, for the mechanism-based inhibitors furafylline and troleandomycin. The other incubation mixtures were preincubated for 5 min at 37°C prior to addition of NADPH (10 mM, final concentration).

The metabolism of laquinimod by cDNA-expressed CYP enzymes was performed by incubating laquinimod (100 μM) with microsomes containing one of the following expressed CYP isoformes from insect cells: CYP1A1, 1A2, 2B6, 2C9, 2C19, 2D6, and 3A4 (1-2 mg of microsomal protein/mL). Microsomes from nontransfected cells were used as controls. Laquinimod metabolism in expressed CYP3A4 was next evaluated over a range of substrate concentrations (5-3000 μM) and kinetic parameters for formation of metabolites were obtained.

To study the effect of laquinimod on the metabolism of ethinyl estradiol and prednisolone, incubations were performed with ethinyl estradiol (100 μM) or prednisolone (100 μM) and laquinimod at a concentration range of 5-3000 μM and the IC_{50} values were calculated. Preliminary experiments were performed to establish conditions for reasonable substrate consumption and time and protein linearity of metabolite formation from ethinyl estradiol and prednisolone.

To determine the effect of known CYP3A4 substrates on the metabolism of laquinimod, the compound (200 μM) was incubated with one of the following compounds; ketoconazole (0.01-20 μM), troleandomycin, (0.5-500 μM), erythromycin (1-750 μM), prednisolone (1-1000 μM) or ethinyl estradiol (1-400 μM) with 3 mg/ml protein for 10-20 min and IC_{50} values were calculated. Concentrations of the different CYP3A4 substrates were chosen based on data from the literature and preliminary experiments to determine a concentration range for each compound, covering no inhibition to almost complete inhibition. The mechanism-based inhibitors troleandomycin and erythromycin were preincubated for 10 min at 37°C, with NADPH prior to addition of laquinimod.

HPLC analyses. Quantification of laquinimod and its metabolites were obtained with an HPLC-system. The samples were injected (50-100 μL) into the chromatographic system and separated on a Reversed Phase Symmetry C18 column, 5 μm, (3.9x150 mm) with RP Symmetry C18 guard column (Waters Associates) and linear gradient system from 20:10:70 to 90:10:0 (acetonitrile: 1M phosphoric acid: water). The analyses were performed at room temperature at a flow of 1.0 mL/min. Radioactivity was detected by a flow detector (Flo-One β, Packard Instrument Co., Meriden CT, USA), with a 0.5 mL flow cell. The mobile phase was mixed post column with Ultima flo AP (Packard Instrument Co., Meriden CT, USA) at a flow of 3 mL/min.

Ethinyl estradiol and metabolites were quantified using an HPLC system (Fernandez et al., 1993) and radiochemical detection. Prednisolone and metabolites were quantified by UV detection at 254 nm. Prednisolone was analysed by HPLC with an isocratic system of 25 % tetrahydrofuran in water.

Calculations and kinetic analysis. The metabolite peak areas from HPLC analysis were converted into pmol and expressed as in relation to mg protein /min or nmol P450/min.

The nature of laquinimod microsomal metabolism was characterised graphically by Eadie-Hofstee plots for the different metabolites and the kinetic parameters K_M, V_{max} and intrinsic clearance (CL_{Int}) were estimated by non-linear regression of the saturation curve by the non-linear procedure PROC NLIN in the SAS statistical software (SAS/STAT, 1989).

CL_{Int} was estimated as V_{max}/K_M using either the one-site or the two-site Michaelis-Menten model:

$$V = V_{max} \bullet S / K_M + S + CL_2 \bullet S$$

Correlation coefficients (r^2) were calculated using linear regression analysis.

Formation of metabolites was plotted versus inhibitor concentration. The IC_{50} values were calculated by non-linear regression of the plot of metabolite formation versus inhibitor concentration, using the one site competition model in PRISM, GraphPad, version 2.0 (GraphPAD Software, Inc. San Diego, USA).

Results

Kinetics of laquinimod metabolism. Laquinimod was metabolised by human liver microsomes and at least six primary metabolites, M1-M6, were detected. A representative HPLC chromatogram is shown in Fig.1. Identification of M1-M6 by mass spectrometry (data not shown) demonstrated hydroxylation of laquinimod in the phenyl ring (M1) and in the quinoline moiety (M2-M4). Furthermore, laquinimod was N-dealkylated in the quinoline part of the molecule (M5) and at the aniline nitrogen (M6). The formation of metabolites was NADPH-dependent, linear with time and protein concentration (data not shown). To determine the Michaelis-Menten parameters for the formation of the primary metabolites, laquinimod at concentrations, 5-3000 µM, was incubated for

20 min with microsomes. The apparent K_M, V_{max} and CL_{Int} are shown in Table 1 and the saturation curves in Fig. 2. The metabolite formation exhibited in general single enzyme Michaelis-Menten kinetics with K_M in the range 0.09-1.9 mM and V_{max} from 22-120 pmol/mg/min for M1-M6. However, the formation of M4 displayed biphasic kinetics with a second CL term that was not saturated over the substrate concentration range used in this study.

Hydroxylation in the quinoline moiety was the major pathway, representing 66 % of total metabolism, whereas the N-demethylation, N-deethylation and the hydroxylation in the aniline part of the molecule constituted about 19, 10, and 4 %, respectively (Fig. 3).

Correlation of laquinimod metabolism towards other specific CYP activities across a panel of human livers. Laquinimod metabolism was determined in microsomal preparations from 15 human livers, which have been characterised with respect to different CYP marker activities. The rate of metabolite formation varied approximately five-fold among the samples (data not shown). Correlations between the rate of formation of M1-M6 and different CYP marker activities are demonstrated in Table 2. A strong correlation (r^2 = 0.90-0.94) between the formation of metabolite M2-M5 and testosterone 6β-hydroxylation (CYP3A4 activity) was found. Likewise, the formation of metabolite M1 and M6 was also correlated to testosterone 6β-hydroxylation (r^2= 0.63 and 0.60 respectively). A notable correlation was also found between M1-M6 and CYP2A6 and CYP2C8 activity (r^2 = 0.46-0.69). However, when the activities for the specific CYP enzymes were compared with each other, correlations were found between CYP3A4/5 and CYP2A6 and 2C8; r^2 = 0.62-0.64. Therefore, the contribution of these enzymes to the metabolism of laquinimod cannot be sufficiently evaluated from these data. No significant correlation for the formation of primary

metabolites was obtained to other CYP activities. A good correlation was obtained toward total CYP content and the formation of laquinimod metabolites (r^2 = 0.43-0.74).

Inhibition of laquinimod metabolism. Competitive and irreversible inhibitors were used to evaluate the CYP enzymes involved in the metabolism of laquinimod. The formation of metabolites M1-M6 was quantified and compared to control incubations (without inhibitor) in human liver microsomes. Inhibitors of CYP3A4, ketoconazole and troleandomycin, were found to inhibit the formation of the primary metabolites, whereas no inhibition was seen with any of the other CYP inhibitors used in this study (Fig. 4). Contrary, quinidine was found to stimulate the formation of M2-M3 and M5 to some degree. Furthermore the formation of metabolite M6 was enhanced in incubations with furafylline.

Metabolism of laquinimod in recombinant expressed CYP enzymes. Laquinimod, at 100 μM, was incubated with the cDNA expressed CYP enzymes, CYP1A1, 1A2, 2B6, 2C9, 2C19, 2D6, and 3A4 (Fig. 5). The formation of the major primary metabolites was preferentially catalysed by CYP3A4 and a similar metabolite pattern was produced by incubation with CYP3A4 as by human liver microsomes. Enzyme kinetic parameters with K_M in the range 0.04-1.7 mM and V_{max} of 0.21-1.0 nmol metabolite/nmol CYP3A4/min were obtained in expressed CYP3A4 (Table 3). The formation of both M3 and M4 was found to be catalysed to some degree by CYP1A1. Furthermore,

M3 was also detected in incubations with CYP2C19. However, the involvement of these enzymes in the metabolism of laquinimod needs to be further evaluated.

The effect of laquinimod on the metabolism of ethinyl estradiol and prednisolone. Incubation of ethinyl estradiol (100 μM) with various concentrations of laquinimod within the range 5-3000 μM resulted in a decreased metabolism of ethinyl estradiol. An IC_{50}-value for laquinimod of 154 μM for the inhibition of ethinyl estradiol metabolism was calculated. Incubation of prednisolone (100 μM) with various concentrations of laquinimod within the range of 5-3000 μM did not affect the metabolism of prednisolone.

The effect of CYP3A4 substrates on the metabolism of laquinimod. Laquinimod was incubated with known CYP3A4 substrates (ketoconazole, troleandomycin, erythromycin, prednisolone, ethinyl estradiol) at concentrations at which no inhibition to total inhibition occurred.

All the studied compounds were found to inhibit the formation of the laquinimod metabolites, M1-M6 (Table 4). The strongest inhibition of laquinimod primary metabolism was demonstrated for ketoconazole with an IC_{50} value of 0.2 μM, followed by troleandomycin, erythromycin, prednisolone and ethinyl estradiol with IC_{50} values of 11, 24, 87, and 235 μM, respectively.

Discussion

In the present study liver microsomal preparations from humans were used to establish the enzymatic basis for the primary metabolism of laquinimod, a new immunomodulator, in man. Laquinimod was metabolised to the primary hydroxylated metabolites M1-M4, and dealkylated metabolites M5 and M6. The metabolite formation exhibited single enzyme Michaelis-Menten kinetics with K_M in the range 0.09-1.9 mM and V_{max} from 22-120 pmol/mg/min for M1-M6. The broad range of K_M values obtained for different metabolic pathways produced by a single enzyme has previously been described for other compounds for instance midazolam, which is metabolized by CYP3A4 to metabolites with very different K_M values (Khan et al. 2002).

Hydroxylation at different sites in the quinoline moiety (M2-M4) was the major pathway (66%) followed by N-demethylation (19%), N-deethylation (10%) and hydroxylation in the para position of the phenyl ring (4%). One of the quinoline hydroxylated metabolites (M2) was also found to be dehalogenated, which might be explained by the fact that some of the rearrangement reactions during CYP catalysed oxidation of aromatic rings involves migration of the geminal hydrogen atom. This displacement, known as the “NIH shift” may also affects lower halogenes such as chloro-substituents (Safe et al., 1976). It remains to be clarified if any of the laquinimod metabolites display pharmacologic or toxicologic activity. However, metabolites formed by aniline-N dealkylation of structurally related compounds have been shown to enhance cell-mediated immunity in animal models (Eriksoo et al., 1985).

The strong correlation observed between the formation rate of the major metabolites, M2-M5 (0.9-0.94), M1, M6 (r~0.6) and 6β-hydroxylation of testosterone (CYP3A4) in the panel of human liver microsomes, implies that both hydroxylation and demethylation of laquinimod are mediated mainly through enzymes in the CYP3A family.

A good correlation was also obtained toward total CYP content, which is in accordance with the relatively high abundance of CYP3A4 in the human liver (Shimada et al., 1994). Furthermore, a five-fold variation in metabolism of laquinimod across the panel is also in good agreement with the large variability reported for CYP3A4 activity within a general population (Wilkinson, 1996).

Moreover, the formation of major metabolites was significantly inhibited by ketoconazole and troleandomycin, specific inhibitors of CYP3A4, which, further support the role of CYP3A4 in the metabolism of laquinimod. Interestingly, the formation of some of the metabolites (M2-M3, M5) was stimulated by co-incubations with quinidine. Both homotropic and heterotropic cooperativity have been described for CYP3A4 (Ekins et al., 1998, Tang and Stearns, 2001). Quinidine has been found to stimulate (for example) the CYP3A4 catalysed metabolism of warfarin in human liver microsomes and hepatocytes (Ngui et al., 2001). Quinidine was also found to stimulate the metabolism of diclofenac both *in vitro* and *in vivo* in rhesus monkeys (Tang and Stearns, 2001, Ngui et al. 2000). *In vivo* examples of heterotropic cooperativity are however rare and the importance of a possible stimulation of the laquinimod metabolism *in vivo* remains to be further studied.

Investigation of secondary metabolism of laquinimod was beyond the scope of the presented study. However, preliminary experiments have demonstrated that the N-dealkylated metabolite, M6, is further rapidly metabolised by hydroxylation in the aniline part of the molecule. By using recombinantly expressed enzymes the reaction was found to be catalysed by enzymes in the CYP1A and to a minor extent, the CYP3A family (data not shown). The enhanced level of M6 after co-incubations with laquinimod and furafylline, a specific CYP1A inhibitor, also supported the role of CYP1A enzymes in the further metabolism of M6.

Recombinantly expressed CYP3A4 was found to produce the whole pattern of primary metabolites from laquinimod. Furthermore, kinetic parameters obtained with recombinant CYP3A4 demonstrated K_M values of the same order of magnitude as in human microsomes.

However, the formation of the hydroxylated metabolites M3 and M4 was also catalysed by CYP1A1. CYP1A1 is not constitutively expressed in the liver but inducible by variety of compounds (cruciferous, cigarette smoke, PAHs, dioxins but also drug compounds such as antimalarials and the benzamidazols. (Fuhr, 2000, Fontaine et al., 1999) and a minor role for CYP1A1 in the laquinimod metabolism can not be excluded. The hydroxylated metabolite M3 was also formed in incubations with CYP2C19. Taken together, a very small role of other CYP enzymes in laquinimod metabolism can not be ruled out completely but the significance of correlation with CYP3A4 activity and laquinimod metabolism integrated with the inhibition results strongly support a principal role for CYP3A4 in the metabolism of laquinimod. When comparing the metabolism of laquinimod in the present study with that of the chemically related compound, roquinimex, (Tuvesson et al. 2000) important similarities are demonstrated. Both compounds are metabolized through a low affinity process by CYP3A4 to hydroxylated and dealkylated metabolites

There are a number of drugs that are metabolised preferentially by enzymes in the CYP3A family and it has been reported that the enzyme may be involved in the metabolism of as much as 50 % of drugs used in humans. This includes several classes of drugs such as calcium channel antagonist, immunosuppressant agents, cholesterol-lowering agents, non-sedating antihistamines, benzodiazepines and macrolide antibiotics (Shou et al., 1994). Thus, identifying CYP3A4 as a major enzyme in the primary metabolism of a compound may be of clinical importance with regard to drug-drug interactions.

The low affinity between laquinimod and the enzyme (high K_M), demonstrated in the present study will reduce the risk for competitive inhibition of the metabolism of other CYP3A4 substrates. However, possible effects of laquinimod on the metabolism of ethinyl estradiol, the main active component in most oral contraceptives and prednisolone, a glucocorticoid widely used as a complement in MS treatment, were studied *in vitro*. The mutual inhibition between laquinimod and ethinyl estradiol demonstrated an IC_{50} value of 154 μM laquinimod inhibition of ethinyl estradiol, while no effects were seen on prednisolone metabolism. Preliminary estimates from ongoing clinical studies demonstrate a C_{max} level at steady-state of below 5 μM of laquinimod when doses of 0.05 up to 2.4 mg daily were given to healthy volunteers or patients. This level of laquinimod in plasma is far below the calculated IC_{50}-value of 154 μM for inhibiting the ethinyl estradiol metabolism.

In vitro studies were undertaken to study the potential of a number of drugs, known to be CYP3A4 substrates, to influence the metabolism of laquinimod. Incubations were performed in order to determine IC_{50} values for the inhibition of the laquinimod metabolism. The strongest inhibition of laquinimod primary metabolism was demonstrated for ketoconazole, with an IC_{50} value of 0.2 μM, followed by troleandomycin, erythromycin, prednisolone and ethinyl estradiol with IC_{50} values of 11, 24, 87, and 235 μM, respectively.

Ketoconazole is a relatively specific and potent inhibitor of CYP3A4 mediated metabolism and the IC_{50} value in the present study is in good agreement with what has been reported for ketoconazole inhibition of other CYP3A4 substrates (Wang et al., 1999). The IC_{50} values obtained for

troleandomycin and erythromycin in the present study are also in accordance with reported data (Echizen and Tanizaki, 2000, Zhao et al.,1999).

To predict possible drug-drug interactions *in vivo*, the *in vitro* effects of the studied drugs (IC_{50} value) have to be considered in the light of clinically relevant concentrations. The unbound concentration of inhibitor around the metabolising enzyme in the liver is one of the key factors determining the extent of drug-drug interactions *in vivo*. However, for practical reasons the unbound concentration of drug in plasma is generally considered to correspond to the concentration in the liver and therefor used in predictions of drug-drug interactions *in vivo*.

Plasma levels of ketoconazole during clinical use generally exceed 1-3 μM (Greenblatt et al., 1998). Since the calculated IC_{50} value for inhibition of laquinimod primary metabolism by ketoconazole is considerably lower than typical clinical plasma concentrations inhibition of laquinimod metabolism is likely to be of clinical importance.

After a single dose of erythromycin (400 mg/kg) a C_{max} value of 2.5 μg/ml (3.4 μM) was obtained (Kanazawa et al., 2001). Comparing plasma concentration and the IC_{50} value for erythromycin in the present study clinically relevant drug-drug interactions seems unlikely. The macrolide antibiotics there among erythromycin and troleandomycin are mechanism-based inhibitors of CYP3A4 (Yamano et al., 2001). The degree of drug-drug interaction caused by these compounds is considered to depend on the concentration of inhibitor as well as the contact time of inhibitor and enzyme, which may be considered in a clinical situation.

In a recent study, plasma levels of 52.5 ng/L (0.18 nM) and 0.96 μM of ethinyl estradiol and prednisolone, respectively were determined in women taking either oral contraceptives (30 μg ethinyl estradiol per day) or repeated oral doses of 20 mg of prednisolone per day (Seidegård et al., 2000). Thus, any drug-drug interaction of ethinyl estradiol and prednisolone with laquinimod seems unlikely to occur when the different plasma levels are compared with the IC_{50} values for the two compounds obtained in the present study.

In conclusion, the present study demonstrates that laquinimod, a novel immunomodulator, is a low affinity substrate for CYP3A4 in man. Considering the low affinity between laquinimod and the enzyme together with plasma concentrations achieved *in vivo* the likelihood of an *in vivo* interaction between laquinimod and other CYP3A4 metabolised drugs in humans might be negated. However, a possible influence of other drugs on the metabolism of laquinimod in man, which may affect the clearance of the compound, can not be excluded. Inhibitory effects on the laquinimod primary metabolism of potent and specific inhibitors of CYP3A4 such as ketoconazole, is anticipated and should be considered in the continued clinical program for laquinimod.

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Legends for figures

FIG. 1 HPLC radiochromatogram of metabolites formed in incubations of laquinimod with human liver microsomes.

FIG. 2 Saturation curves for the formation of metabolites (M1-M6) from laquinimod in human liver microsomes.

FIG. 3 Formation of primary metabolites of laquinimod in human liver microsomal incubations. Heavy arrows denote major metabolites, light arrows represent minor metabolites.

- Site of metabolism.

FIG. 4 Effect of the CYP inhibitors furafylline (20 μM), coumarin (25 μM), diclofenac (200 μM), sulfaphenazole (50 μM), quinidine (25 μM), chlorzoxazone (50 μM), ketoconazole (2 μM) and troleandomycin (100 μM) on the formation rate (% of control) of laquinimod metabolites M1-M6, expressed as a sum, at 200 μM laquinimod in human microsomes. Data are the average of two determinations.

FIG. 5 Metabolism of laquinimod (100 μM) by cDNA expressed CYP enzymes.

TABLE 1

Enzyme kinetic parameters for the formation of laquinimod metabolites in human liver microsomes

Incubations were conducted using laquinimod concentrations from 5 to 3000 µM. Reactions were linear with respect to protein concentrations and time. Results are presented as obtained values from a non linear regression analysis (SE).

Metabolite	**V_{max} (pmol/mg /min)**	**K_M (mM)**	**CL_{Int} (µL/mg/min)**	**CL_{Int2} (µL/mg/min)**
M1	120(57)	1.9 (1.1)	0.065(0.012)	
M2	107(19)	0.93 (0.23)	0.12(0.012)	
M3	51(6.2)	0.09(0.02)	0.59(0.11)	
M4	28 (7.4)	0.11(0.03)	0.26(0.02)	0.03 (0.2)[a)]
M5	88 (6.2)	0.31(0.04)	0.29(0.02)	
M6	22 (3.0)	0.15(0.04)	0.15(0.03)	

a) not saturated over the substrate concentration range studied (5-3000 µM)

TABLE 2

Correlation of laquinimod metabolites (M1-M6) formation with total CYP content and with different CYP activities across a panel of human liver microsomes (n=15)

		Correlation (r^2)					
Marker activity	**CYP**	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**
Total CYP content		0.54*	0.74*	0.72*	0.68*	0.62*	0.43
7-Ethoxyresorufin O-dealkylation	**1A2**	a)	0.02	0.03	0.01	0.03	0.15
Coumarin 7-hydroxylation	**2A6**	0.46	0.59*	0.56*	0.55*	0.46	0.50
7-Ehoxy-4-trifluoromethyl-coumarin O-deethylation	**2B6**	0.20	0.31	0.36	0.36	0.40	0.09
Paclitaxel 6α-hydroxylation	**2C8**	0.60*	0.69*	0.69*	0.65*	0.55*	0.63*
Tolbutamide methyl-hydroxylation	**2C9**	0.21	0.13	0.14	0.21	0.13	0.34
S-Mephenytoin 4-hydroxylation	**2C19**	0.09	0.15	0.19	0.21	0.14	0.07
Dextromethorphan O-demethylation	**2D6**	0.04	0.09	0.07	0.05	0.07	0.05
Chlorzoxazone 6-hydroxylation	**2E1**	0.02	0.01	0.01	a)	0.03	0.01

Testosterone	**3A4/5**	0.63*	0.94*	0.91*	0.90*	0.92*	0.60*
6β-hydroxylation							
Lauric acid 12-hydroxylation	**4A9/11**	0.08	0.22	0.20	0.18	0.14	0.24

a) Not detectable

* $p < 0.0001$

TABLE 3

Enzyme kinetic parameters for the formation of laquinimod metabolites by recombinant CYP3A4

Incubations were conducted using laquinimod concentrations from 5 to 3000 μM and 200 pmol CYP3A4/mL. Reactions were linear with respect to enzyme concentration and time. Results are presented as obtained values from a non linear regression analysis (SE).

Metabolite	**K_M (mM)**	**V_{max} (pmol/mg/min)**
M1	1.7 (0.8)	1.0 (0.2)
M2	a)	a)
M3	0.04 (0.02)	0.21 (0.02)
M4	0.45 (0.21)	0.42 (0.07)
M5	0.39 (0.17)	0.71 (0.10)
M6	0.98 (0.24)	1.0 (0.1)

a) Below limit of quantification

TABLE 4

Effects of various drugs on laquinimod primary metabolism

Studies were conducted using a laquinimod concentration of 200 μM and various concentrations of the studied drug compounds.

	IC_{50} (μM)
Ketoconazole	0.21
Troleandomycin	11
Erythromycin	24
Ethinyl estradiol	235
Prednisolone	87

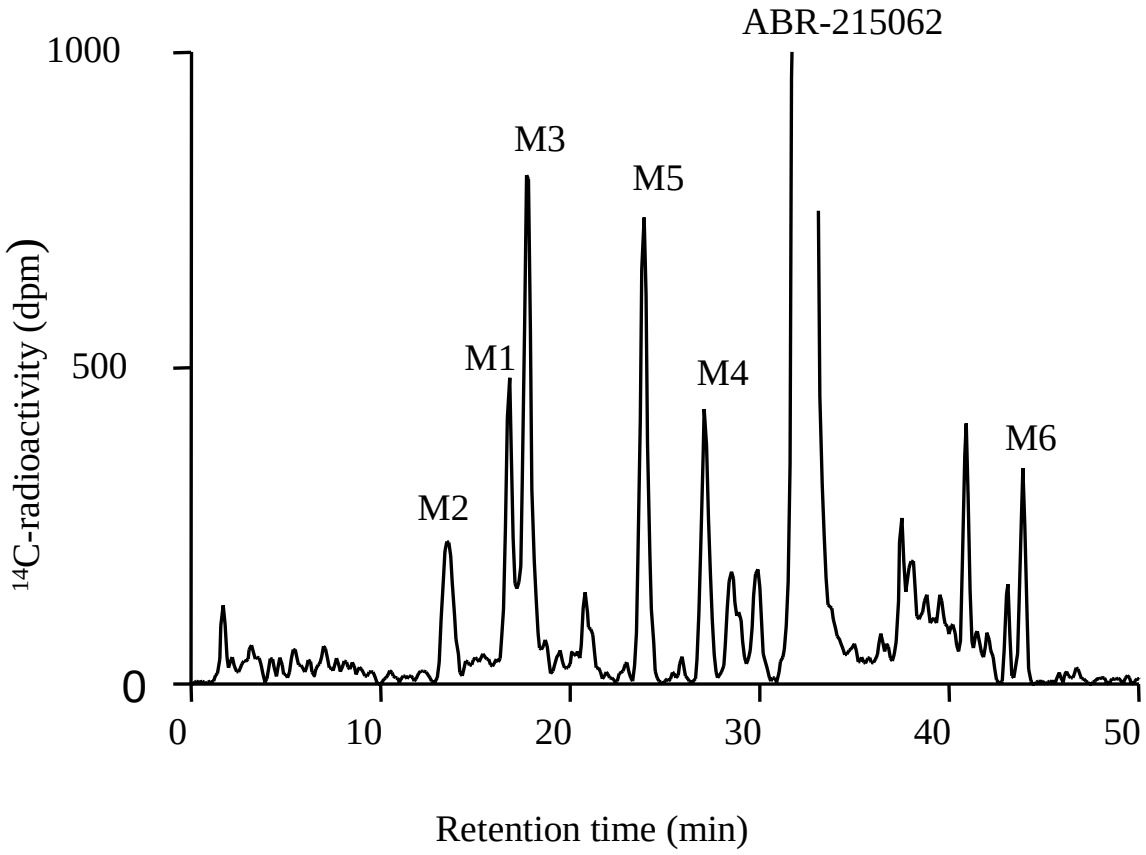


FIG.1.

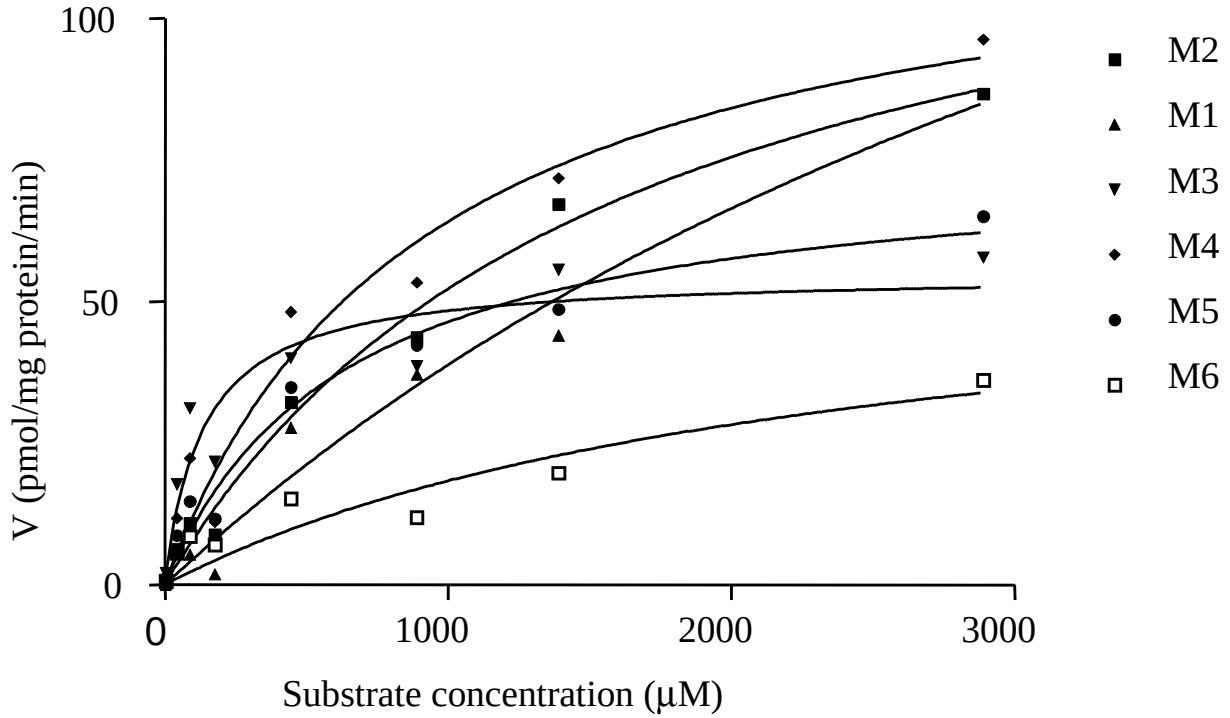
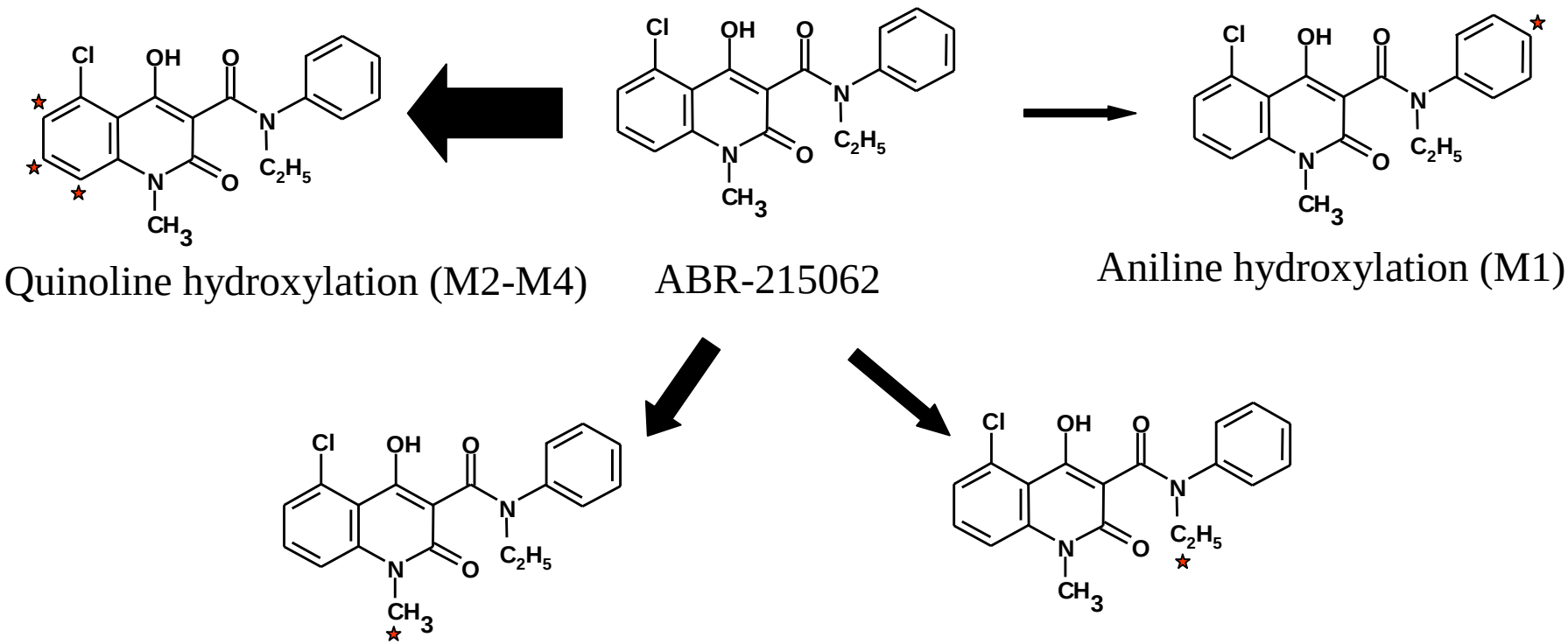


FIG.2.

FIG.3.



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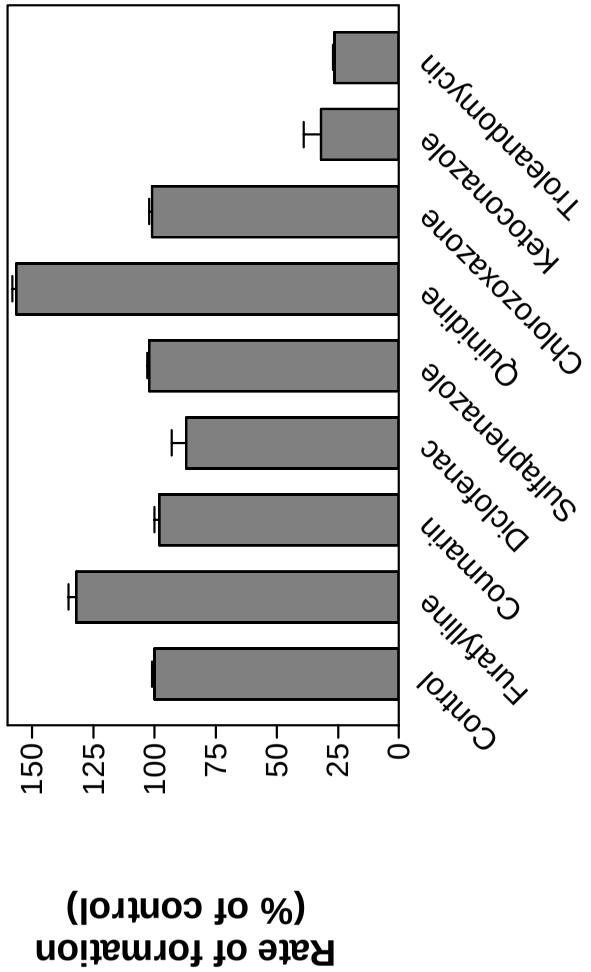

FIG.4.

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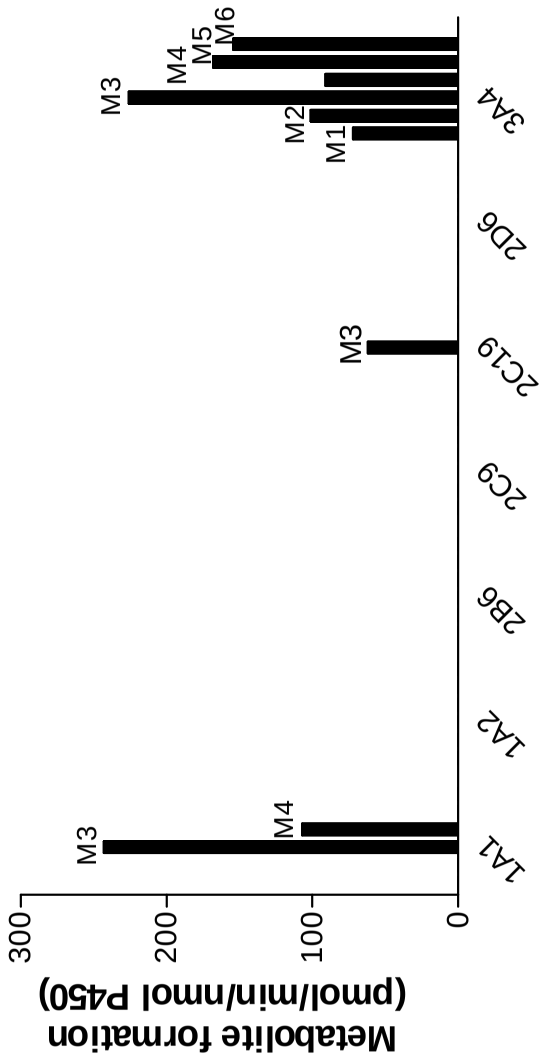

FIG.5.