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OMEGA-OXIDATION OF VERY LONG-CHAIN FATTY ACIDS IN HUMAN LIVER MICROSOMES: IMPLICATIONS FOR X-LINKED ADRENOLEUKODYSTROPHY

Robert-Jan Sanders, Rob Ofman, Marinus Duran, Stephan Kemp, Ronald J.A. Wanders*
From the department of Genetic Metabolic Diseases, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands

Running title: Omega-oxidation of VLCFA

Address correspondence to: Prof. Dr. Ronald J.A. Wanders, Laboratory Genetic Metabolic Diseases (Room F0-224), Academic Medical Center, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands, Phone: +31-20-5665958, Fax: +31-20-6962596, E-mail: r.j.wanders@amc.uva.nl

X-linked adrenoleukodystrophy (X-ALD) is a severe neurodegenerative disorder biochemically characterized by elevated levels of very long-chain fatty acids (VLCFA). Excess levels of VLCFAs are thought to play an important role in the pathogenesis of X-ALD. Therefore, therapeutic approaches for X-ALD are focused on the reduction or normalization of VLCFAs. In this study, we investigated an alternative oxidation route for VLCFAs, namely ω-oxidation. The results described in this paper show that VLCFAs are substrates for the ω-oxidation system in human liver microsomes. Moreover, VLCFAs were not only converted into ω-hydroxy fatty acids, but they were also further oxidized to dicarboxylic acids via cytochrome P450 mediated reactions. High sensitivity towards the specific P450 inhibitor 17-octadecynoic acid suggested that ω-hydroxylation of VLCFAs is catalyzed by P450 enzymes belonging to the CYP4A/ F subfamilies. Studies with individually expressed human recombinant P450 enzymes revealed that two P450 enzymes, i.e. CYP4F2 and CYP4F3B, participate in the ω-hydroxylation of VLCFAs. Both enzymes belong to the cytochrome P450 4F subfamily and have a high affinity for VLCFAs. In summary, this study demonstrates that VLCFAs are substrates for the human ω-oxidation system and for this reason stimulation of the *in vivo* VLCFA ω-oxidation pathway may provide an alternative mode of treatment to reduce the levels of VLCFAs in patients with X-ALD.

In mammalian cells, fatty acid oxidation plays a major role in the production of energy particularly in the heart and skeletal muscle, and is the main energy source during periods of fasting. Both mitochondria and peroxisomes are capable of degrading saturated fatty acids via β-oxidation. Short-, medium- and long-chain saturated fatty acids are degraded predominantly by mitochondria, whereas very-long chain fatty acids (VLCFA, >22 carbons) are β-oxidized exclusively in peroxisomes (1;2). Moreover, peroxisomes also metabolize certain branched chain fatty acids, bile acid precursors, eicosanoids and dicarboxylic acids (3).

X-linked adrenoleukodystrophy (X-ALD: MIM 300100), the most common peroxisomal disorder, is a progressive neurodegenerative disease that affects the cerebral white matter, spinal cord, peripheral nerves, adrenal cortex and testis (4). X-ALD is caused by mutations in the ABCD1 gene that encodes ALDP, an ATP-binding cassette transporter located in the peroxisomal membrane with an unknown function (5). Biochemically, X-ALD is characterized by elevated levels of saturated and monounsaturated VLCFAs in plasma and tissues, due to the impaired β-oxidation of VLCFAs in peroxisomes (6-8). Since the pathogenesis of X-ALD is probably due to the increased levels of VLCFAs, correction of VLCFA levels is one of the primary objectives in therapeutic approaches. These include gene replacement therapy, bone marrow transplantation, lovastatin treatment, inhibition of VLCFA biosynthesis by mono-unsaturated fatty acids, notably oleic acid (C18:1ω9) and erucic acid (C22:1ω9) (Lorenzo's Oil), and induction of the expression of ALDP-related protein (ALDR) (see ref (4) for an overview). Despite the increasing knowledge about X-ALD, there is currently no effective therapy for this disease.

An alternative method to degrade VLCFAs would be the ω-oxidation followed by β-oxidation of the dicarboxylic acids produced. Different fatty acids are known to undergo ω-oxidation, but no data in literature is available with respect to the ω-oxidation of VLCFAs (9). The first step in ω-oxidation of fatty acids involves the

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conversion of the ω-methyl group of the fatty acid into an ω-hydroxyl group. This reaction is carried out by one or more cytochrome P450 enzymes mainly belonging to the CYP4 subfamily and requires NADPH and molecular oxygen (9;10). Subsequently, the ω-hydroxy fatty acid may be oxidized further into a ω-carboxylic acid either via an NAD^+-dependent alcohol and aldehyde dehydrogenase system or via a cytochrome P450 mediated route (11-14). Finally, dicarboxylic acids can be β-oxidized in peroxisomes and/ or mitochondria to shorter-chain dicarboxylic acids followed by excretion into the urine (15;16). Accumulation of dicarboxylic acids has not been detected in X-ALD patients, whereas in patients with a peroxisomal biogenesis disorder (PBD), elevated levels of medium- and long-chain dicarboxylic acids were found in urine (17). Furthermore, β-oxidation of long-chain dicarboxylic acids was normal in fibroblasts from X-ALD patients and deficient in fibroblasts from PBD patients (15). These studies indicate that peroxisomes play an essential role in dicarboxylic acid degradation and that this metabolic route does not require ALDP. Under normal physiological conditions, fatty acid ω-oxidation is a minor oxidation pathway that accounts for 5-10 % of total fatty acid oxidation in the liver, but the expression levels of many cytochrome P450s can be induced by a variety of different agents (18;19). Therefore, if VLCFAs can indeed undergo ω-oxidation, then stimulation of this activity could be a means to reduce the levels of these fatty acids in patients with X-ALD.

In this study, we investigated the VLCFA ω-oxidation capacity of human liver microsomes. Until now, ω-oxidation of VLCFAs has not been studied in humans and none of the enzymes potentially involved in this system have been characterized. We have studied the ω-oxidation pathway for several saturated fatty acids known to be of relevance to X-ALD, which includes docosanoic acid (C22:0), tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0).

EXPERIMENTAL PROCEDURES

Materials - Pooled human liver microsomes and the recombinant human P450-containing insect cell microsomes and control microsomes (Supersomes™) used in this study were purchased from BD Bioscience/Gentest (Woburn, MA). Cytochrome P450 content of Supersomes™ as provided by the manufacturer were: CYP2E1 (588 pmol/ mg), CYP2J2 (185 pmol/ mg), CYP3A4 (606 pmol/ mg), CYP4A11 (120 pmol/ mg), CYP4F2 (556 pmol/ mg), CYP4F3A (33 pmol/ mg), CYP4F3B (435 pmol/ mg), CYP4 F12 (213 pmol/ mg) and control (not detectable). 22-Hydroxy-docosanoic acid (ω-hydroxy-C22:0) and hexacosanedioic acid (C26:0-DCA) were purchased from Larodan Fine Chemicals (Malmö, Sweden). Sulphaphenazole, quinidine, ketoconazole, furafylline, trimethoprim, diethyldithiocarbamate (DDC) and 17-octadecynoic acid (17-ODYA) were purchased from Sigma Aldrich (St Louis, MO, USA). N, O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). All other chemicals used were of analytical grade.

Enzymatic assay for VLCFA ω-hydroxylation - The experimental conditions used in this paper to study the hydroxylation of different VLCFAs were adapted from previous experiments with minor modifications (14). Briefly, incubations were carried out for 30 min at 37 °C in a reaction mixture that contained Tris buffer pH 8.4 (100 mM), protein (50 µg), α-cyclodextrin (1 mg/ml) and NADPH (1 mM) in a total volume of 200 µl, unless otherwise stated. The reaction was initiated by addition of the fatty acid at a final concentration of 200 µM and terminated by addition of 1 ml hydrochloric acid to a final concentration of 1.7 M. The reaction products were extracted as described previously and analyzed by electrospray ionization mass spectrometry (8;14).

Characterization of ω-hydroxy fatty acids by GC-MS - Incubations were carried out under the same conditions as described above in a total volume of 2 ml. After acidification of the mixture, fatty acids were extracted four times with 5 ml hexane and subsequently dried under a stream of nitrogen. Prior to gas chromatography-mass spectrometry (GC-MS), the residue was incubated with 40 µl BSTFA containing 1% TMCS and 10 µl pyridine at 80 °C for 1 h. After derivatisation, the mixture was used directly for GC-MS analysis on a Hewlett-Packard 6890 gas chromatograph coupled

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to a Hewlett-Packard 5973 mass-selective detector (Palo Alto, CA). The samples (1 µl) were injected in the splitless mode (Hewlett Packard 7683 injector) and separated on a CP-Sil 5 CB-MS low bleed column (25 m × 0.25 mm × 0.30 µm : Chrompack, Middelburg, The Netherlands). The oven temperature was programmed as follows: 2 min at 85 °C followed by a linear increase of 20°C per minute to 320°C and held at 320°C for 10 min. The identities of the reaction products were verified by taking mass spectra in the scanning electron impact mode. The single ion monitoring mode was applied for the detection of the respective $(M-15)^+$ ions, with m/z 485, 513 and 541 of the TMS derivatives of ω and (ω-1)-hydroxy C22:0, C24:0 and C26:0, respectively. The $(M-15)^+$ ions, with m/z 499, 427 and 555 were monitored for the $(M-15)^+$ ions of the TMS derivatives of the dicarboxylic acid of C22:0, C24:0 and C26:0, respectively. The ratio of ω and (ω-1) hydroxy-fatty acids was calculated by comparing the peak areas of the single ion current of the ω-hydroxy fatty acid and dicarboxylic acid, with the peak area of the (ω-1)-hydroxy fatty acid.

Curve-fitting procedure - The best-fit curves to the product formation data in human liver microsomes were obtained from the kinetic equation for the dual-enzyme Michaelis-Menten model,

Eq. 1
$$V = \frac{V_{max1} \cdot S}{K_{m1} + S} + \frac{V_{max2} \cdot S}{K_{m2} + S}$$

where Km1 and Km2 are the high and low affinity Michaelis-Menten constants, respectively, and V_{max1} and V_{max2} are the corresponding maximal catalytic activities. The best-fit curves to the product formation with individually expressed human recombinant P450 enzymes were calculated using a cooperative single-enzyme model with two binding sites (Eq. 2) in which product can be formed either from the single-substrate-bound form or from the two-substrate-bound form of the enzyme (20):

Eq. 2
$$V = \frac{\left(\frac{V_{max1} \cdot S}{K_{m1}} + \frac{V_{max2} \cdot S^2}{K_{m1} \cdot K_{m2}} \right)}{\left(1 + \frac{S}{K_{m1}} + \frac{S^2}{K_{m1} \cdot K_{m2}} \right)}$$

The values of the kinetic parameters were calculated by fitting the experimental data in the appropriate enzyme model using the IGOR Pro 5 software program (Wavemetrics).

RESULTS

Optimization of the VLCFA hydroxylation assay - Previous studies have demonstrated that C22:0 is a substrate for the ω-oxidation system in rat liver microsomes (14). To study the ω-hydroxylation capacity of human liver microsomes for VLCFAs, the hydroxylation assay was optimized for the ω-oxidation of C26:0. To this end, human microsomal protein was incubated in a buffered medium containing NADPH and α-cyclodextrin to solubilize C26:0. The production of ω-hydroxy-C26:0 was linear with time up to 30 min (Figure 1A). The effect of pH on the hydroxylation activity was determined using a combined buffer system that contained 100 mM HEPES and 100 mM glycine to cover the pH range 6.6 - 10.5. Figure 1B shows that the formation of ω-hydroxy-C26:0 was maximal at pH 8.4.

Interestingly, the oxidation of C26:0 was not limited to the production of ω-hydroxy-C26:0, but also the corresponding dicarboxylic acid (C26:0-DCA) was detected. The identity of C26:0-DCA was confirmed by two signals that appeared in the electrospray ionization mass spectra which corresponded to its single- and double negative charged state (data not shown), as well as by GC-MS studies (see below). The formation of C26:0-DCA from C26:0 was linear with time, and maximal at the same pH as for the formation of ω-hydroxy-C26:0 (Figure 1A, B). Total product formation was linear with protein up to 60 µg/ ml (Figure 1C). At higher protein concentrations, the production of ω-hydroxy-C26:0 decreased, whereas C26:0-DCA formation increased slightly. Several buffer systems at pH 8.4 were tested: 100 mM Tris, 100 mM Tricine and 100 mM Hepes. Hydroxylation of C26:0 was maximal with Tris as

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buffer (results not shown), which has therefore been used in subsequent experiments.

Kinetic analysis of VLCFA hydroxylation - The enzyme kinetics for the hydroxylation of C22:0, C24:0 and C26:0 were analyzed. To determine apparent Km and Vmax values for the different fatty acids, rates of product formation were determined at different substrate concentrations (Figure 2). Interestingly, at the highest substrate concentrations used, the ratio of ω-hydroxy/ dicarboxylic acid produced decreased with increasing chain-length of the substrate. Furthermore, the rate of total product formation versus substrate concentration of all fatty acids used did not follow simple Michaelis-Menten kinetics. Different models were tested for data fitting. However, the model for dual-enzyme Michaelis-Menten kinetics as described in Experimental Procedures produced the best fit of the data points of Figure 2. The kinetic parameters calculated from the experimental data of each of the different substrates using this model are listed in Table 1. At low substrate concentrations, the highest activity was observed with C22:0 with an apparent Vmax of 0.8 nmol· min^{-1}· mg^{-1}, whereas the highest affinity was observed for C26:0 with an apparent Km of <1μM. The results described in Table 1 show that the highest catalytic efficiency, Vmax/ Km ratio, is observed for C26:0. The hydroxylation efficiency for C24:0 and C22:0 is 10-fold lower as compared to C26:0. Overall, these results demonstrate that VLCFAs are substrates for the human microsomal ω-oxidation system.

Characterization of VLCFA ω-oxidation products - Hydroxylation of VLCFAs by cytochrome P450 enzymes may occur at the ω-position as well as at the adjacent positions (9;21). To determine the position of the hydroxyl-group, reaction products of the incubations with C22:0, C24:0 and C26:0 were analyzed by GC-MS. Three products appeared in the gas chromatogram after incubating human liver microsomes with C26:0 (Fig. 3). The major peak (Fig. 3, peak II) with a retention time of 14.5 min was analyzed by mass spectral analysis and identified as ω-hydroxy-C26:0 (Fig 4A). The fragment at m/z 103 is characteristic of ω-hydroxy fatty acids, representing the terminal $CH_2OSi(CH_3)_3$ moiety (22). The fragments observed in this mass spectrum were in analogy with those of the commercially available 22-hydroxy-C22:0 (data not shown). The minor peak in the GC chromatogram with a retention time of 14.25 min (Fig. 3, peak I) was identified by mass spectral analysis as (ω-1)-hydroxy-C26:0 (Fig. 4B). It was deduced from the mass spectrum that the peak at m/z 117 represents the $CH_3CHOSi(CH_3)_3$ due to cleavage of the molecule adjacent to the hydroxyl group. Hydroxylated products of C26:0 with the hydroxyl-group at other positions than ω and (ω-1) were not detected. In addition, the third peak (Fig. 3, peak III) with retention time of 14.85 min was identified as C26:0-DCA (Fig. 4C). The fragmentation pattern of this compound was identical to that of the commercial available C26:0-DCA (not shown). Product analysis of incubations with C22:0 and C24:0 as substrates resulted in similar patterns of product formation and analogous mass spectra as observed for C26:0 (data not shown). The ratio ω/ (ω-1)-hydroxylation for each substrate is listed in Table1. Apparently, cytochrome P450 enzymes preferably hydroxylate VLCFAs at the methyl-group.

VLCFA-hydroxylation inhibition studies - The first step of the ω-oxidation of fatty acids is catalyzed by cytochrome P450 enzymes. The human cytochrome P450 family consists of at least 57 CYP isoforms that are able to oxidize a broad spectrum of chemical compounds (23). In order to identify the P450 subfamily and/ or the individual CYP isoform that is involved in VLCFA hydroxylation, various inhibitors were tested, which include: sulfaphenazole (selective for CYP2C) (24), quinidine (for CYP2D6) (24), ketoconazole (for CYP3A) (25), troleandomycin (for CYP3A4) (24), furafylline (for CYP1A2) (26), DDC (for CYP2E1) (26), trimethoprim (for CYP2C8) (27) and 17-ODYA (for CYP4A/F) (10;28). In Figure 5, the effect of these inhibitors on the ω-oxidation of C26:0 is shown. Hydroxylation of C26:0 was inhibited markedly by 17-ODYA already at the lowest concentration used (1 μM) and no ω-oxidation products were detected at the highest concentration of inhibitor used (100 μM). In the presence of 1 μM troleandomycin, product formation was decreased by 20%. DDC and ketoconazole, inhibitors of CYP2E1 and CYP3A, respectively, had some

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inhibitory effect at the highest concentration inhibitor used (100 μM). All other inhibitors tested did not affect the hydroxylation of C26:0 to any significant extent.

Identification of P450 VLCFA-hydroxylases - The results from the VLCFA inhibition studies indicated that cytochrome P450 enzymes belonging to the CYP2E1, CYP3A and CYP4A/F subfamily are likely to be involved in the hydroxylation of these fatty acids. To confirm this, several human recombinant P450 enzymes (Supersomes™) were tested for hydroxylation activity towards C26:0. Supersomes™ are microsomes from baculovirus-infected insect cells expressing a single human CYP isoform. These experiments revealed that CYP4F2 as well as CYP4F3B catalyzed the hydroxylation of C26:0 (Figure 6). All other recombinant CYP isoforms tested as well as non-CYP containing Supersomes™ did not hydroxylate C26:0 to any appreciable extent.

Kinetic analysis of VLCFA hydroxylation by CYP4F2 and CYP4F3B - The enzyme kinetics of CYP4F2 and CYP4F3B were analyzed for the hydroxylation of different VLCFAs. Figures 7 and 8 show that hydroxylation of VLCFAs was not limited to the production of the ω-hydroxylated product, but dicarboxylic acids were produced as well. The rate of total product formation versus substrate concentration again did not follow Michaelis-Menten kinetics. The plots were hyperbolic and product formation was not saturable, even at the highest substrate concentration used (200 μM). The corresponding Eadie-Hofstee plots were biphasic (data not shown), indicating apparent multiple-binding site kinetics (20). To determine the kinetic parameters of CYP4F2 and CYP4F3B for the fatty acids tested, different enzyme kinetic models were analyzed. A cooperative single-enzyme model with two binding sites as described in Experimental Procedures resulted in the best fit of the data points of Figures 7 and 8. The kinetic parameters calculated for each of the different substrates are listed in Table 2. Apparent Km values of both enzymes for VLCFAs were in the micromolar range. The highest VLCFA hydroxylation activity was observed for CYP4F3B. Although CYP4F2 was found to be less active towards VLCFAs, Vmax/ Km ratios were similar to those of CYP4F3B.

The reaction products after incubation of CYP4F2 or CYP4F3B with the different fatty acids were analyzed by GC-MS. Both enzymes predominantly hydroxylate the methyl-group of the VLCFAs and to a lesser extend the (ω-1)-group (data not shown). The ω: (ω-1)-hydroxylation ratio for each substrate is listed in Table 2.

DISCUSSION

In this study, we investigated whether the ω-oxidation pathway may provide an alternative oxidation route for VLCFAs. Our results show that these fatty acids are substrates for the ω-oxidation system in human liver microsomes. Moreover, C26:0 was not only converted into ω-hydroxy-C26:0 but also further to its dicarboxylic acid by cytochrome P450 enzymes. Our previous studies on C22:0 ω-oxidation demonstrated the existence of a cytochrome P450 mediated hydroxylation system for the production of dicarboxylic acids in rat liver microsomes (14). The results described in this paper clearly show that in humans a similar pathway is present for the ω-oxidation of VLCFAs. Based on the inhibition studies (Fig. 5) and the experiments with Supersomes™ containing individual human cytochrome P450 enzymes (Fig. 6), we conclude that CYP4F2 and CYP4F3B are able to hydroxylate C26:0. Moreover, both enzymes are able to hydroxylate C26:0 all the way to its dicarboxylic acid (Fig. 7/ 8). CYP4F2 and CYP4F3B have a high affinity for saturated VLCFAs with Km values in the micromolar range and are therefore interesting from a physiological point of view.

CYP4F2 and CYP4F3 are distinct genes and both located on the short arm of chromosome 19. The CYP4F2 and CYP4F3 proteins contain 520 amino acids and they share a 94% identity. Both CYP4F2 and CYP4F3B catalyze the ω-hydroxylation of various eicosanoids including arachidonic acid, prostaglandins and leukotriene B4 (29). These enzymes are expressed predominantly in human liver and kidney, and to lesser extend in brain, testis, skin and various other tissues (29-32). Alternative splicing of the CYP4F3 gene generates two distinct isoforms, CYP4F3A and CYP4F3B, and these proteins

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differ in tissue distribution and biological function (32). CYP4F3A does not contain exon 3 and inactivates leukotriene B_4 by ω-hydroxylation. In contrast, CYP4F3B does not contain exon 4, and has high ω-hydroxylation activity towards arachidonic acid (32-34). The substrate specificity of CYP4F3 is apparently determined by the protein domains encoded by exon 3 (CYP4F3B) and exon 4 (CYP4F3A). Our data is in line with this: CYP4F3B has high ω-hydroxylation activity towards VLCFAs, whereas no product formation was observed with CYP4F3A.

Biochemically, X-ALD is characterized by elevated levels of VLCFAs in plasma and tissues, due to the impaired β-oxidation of these fatty acids in peroxisomes. At present, there is no effective therapy for this disease. Based on the hypothesis that the accumulation of VLCFAs in X-ALD patients may be reduced by stimulating VLCFA ω-oxidation, we have now studied the ω-oxidation of VLCFAs. Long-chain dicarboxylic acids can undergo β-oxidation in X-ALD fibroblasts, whereas fibroblasts from patients with a peroxisomal biogenesis disorder (PBD) were unable to oxidize these compounds (15). Furthermore, accumulation of dicarboxylic acids has not been detected in X-ALD patients, while elevated levels of medium- and long-chain dicarboxylic acids were detected in urine of PBD patients (17;35). These studies indicate that peroxisomes are essential for the degradation of long-chain dicarboxylic acids and that ALDP is not involved in the β-oxidation of these fatty acids.

Expression of both CYP4F2 and CYP4F3 has been detected in tissues that are affected in patients with X-ALD. The tissue distribution of CYP4F3B is quite similar to that of CYP4F2 (29). At present, little information is known in literature about the regulation of gene expression of CYP4F2 and CYP4F3B. Many cytochrome P450 enzymes are under the control of one or more members of the nuclear hormone receptor family, notably PPARα, RAR, RXR, PXR, and CAR (18). Unlike P450 enzymes of the CYP4A subfamily, which are induced by peroxisomal proliferators and hypolipidemic drugs like WY14,643 and clofibrate, the expression of CYP4F enzymes either remains unchanged or is repressed (36;37). Induction studies on the promoter activity of the CYP4F2 gene demonstrated that it may be mediated via RAR/RXRα and PPARα/RXRα (38).

CYP4F2 and CYP4F3B predominantly produce ω-hydroxy-VLCFAs and to a lesser extent their corresponding dicarboxylic acids. Further oxidation of ω-hydroxy-VLCFAs can occur via NAD^+-dependent alcohol- and aldehyde dehydrogenases localized in the cytosol and endoplasmic reticulum. This pathway has been identified for long-chain ω-hydroxy-fatty acids, retinoids and (very-)long-chain aldehydes in the endoplasmic reticulum and cytosol (14;39-42). The enzymes involved in the dehydrogenation of these compounds have a broad substrate specificity (40;42;43). Our previous studies have shown that rat liver microsomes readily oxidize ω-hydroxy-C22:0 to a dicarboxylic acid in a NAD^+-dependent pathway and we therefore postulate that the dehydrogenases involved in this pathway may utilize ω-hydroxy-VLCFAs as well (14). At present, there is no data in literature on the nature of the alcohol- and aldehyde dehydrogenases that are active towards ω-hydroxy-VLCFAs. Future studies are aimed at the characterization of the enzymes involved in the NAD^+-dependent ω-oxidation of ω-hydroxy VLCFAs.

To summarize, VLCFAs are hydroxylated to very long-chain ω-hydroxy and dicarboxylic acids in human liver microsomes. We have identified two cytochrome P450 enzymes that catalyze the ω-hydroxylation of VLCFAs. Both enzymes are members of the 4F subfamily, namely CYP4F2 and CYP4F3B. Future work is aimed at the identification of the regulatory mechanisms involved in the expression of these enzymes. To generate new therapeutic options for patients with X-ALD, we will investigate whether the VLCFA ω-oxidation route can be induced in order to normalize the levels of VLCFAs in affected tissues.

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REFERENCES

1. Wanders, R. J. and Tager, J. M. (1998) *Mol.Aspects Med.* **19,** 69-154
2. Kemp, S., Valianpour, F., Mooyer, P. A., Kulik, W., and Wanders, R. J. (2004) *Clin.Chem.* **50,** 1824-1826
3. Wanders, R. J. (2004) *Mol.Genet.Metab* **83,** 16-27
4. Moser, H. W., Smith, K. D., Watkins, P. A., Powers, J., and Moser, A. B. (2001) X-linked adrenoleukodystrophy. In Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., editors. *The Metabolic and Molecular Bases Of Inherited Disease*, McGraw Hill, New York
5. Mosser, J., Douar, A. M., Sarde, C. O., Kioschis, P., Feil, R., Moser, H., Poustka, A. M., Mandel, J. L., and Aubourg, P. (1993) *Nature* **361,** 726-730
6. Igarashi, M., Schaumburg, H. H., Powers, J., Kishmoto, Y., Kolodny, E., and Suzuki, K. (1976) *J.Neurochem.* **26,** 851-860
7. Singh, I., Moser, A. E., Moser, H. W., and Kishimoto, Y. (1984) *Pediatr.Res.* **18,** 286-290
8. Valianpour, F., Selhorst, J. J., van Lint, L. E., van Gennip, A. H., Wanders, R. J., and Kemp, S. (2003) *Mol.Genet.Metab* **79,** 189-196
9. Okita, R. T. and Okita, J. R. (2001) *Curr.Drug Metab* **2,** 265-281
10. Xu, F., Falck, J. R., Ortiz de Montellano, P. R., and Kroetz, D. L. (2004) *J.Pharmacol.Exp.Ther.* **308,** 887-895
11. Kundu, R. K., Tonsgard, J. H., and Getz, G. S. (1991) *J.Clin.Invest* **88,** 1865-1872
12. Robbins, K. C. (1968) *Arch.Biochem.Biophys.* **123,** 531-538
13. Wakabayashi, K. and Shimazono, N. (1963) *Biochim.Biophys.Acta* **70,** 132-142
14. Sanders, R. J., Ofman, R., Valianpour, F., Kemp, S., and Wanders, R. J. (2005) *J.Lipid Res.* **46,** 1001-1008
15. Ferdinandusse, S., Denis, S., Van Roermund, C. W., Wanders, R. J., and Dacremont, G. (2004) *J.Lipid Res.* **45,** 1104-1111
16. Kolvraa, S. and Gregersen, N. (1986) *Biochim.Biophys.Acta* **876,** 515-525
17. Rocchiccioli, F., Aubourg, P., and Bougneres, P. F. (1986) *Pediatr.Res.* **20,** 62-66
18. Dickins, M. (2004) *Curr.Top.Med.Chem.* **4,** 1745-1766
19. Bjorkhem, I. (1978) *J.Lipid Res.* **19,** 585-590
20. Korzekwa, K. R., Krishnamachary, N., Shou, M., Ogai, A., Parise, R. A., Rettie, A. E., Gonzalez, F. J., and Tracy, T. S. (1998) *Biochemistry* **37,** 4137-4147
21. Adas, F., Salaun, J. P., Berthou, F., Picart, D., Simon, B., and Amet, Y. (1999) *J.Lipid Res.* **40,** 1990-1997
22. Jakobs, C., Bojasch, M., Monch, E., Rating, D., Siemes, H., and Hanefeld, F. (1981) *Clin.Chim.Acta* **111,** 169-178
23. Lewis, D. F. (2004) *Pharmacogenomics.* **5,** 305-318
24. Newton, D. J., Wang, R. W., and Lu, A. Y. (1995) *Drug Metab Dispos.* **23,** 154-158
25. Baldwin, S. J., Bloomer, J. C., Smith, G. J., Ayrton, A. D., Clarke, S. E., and Chenery, R. J. (1995) *Xenobiotica* **25,** 261-270
26. Eagling, V. A., Tjia, J. F., and Back, D. J. (1998) *Br.J.Clin.Pharmacol.* **45,** 107-114
27. Wen, X., Wang, J. S., Backman, J. T., Laitila, J., and Neuvonen, P. J. (2002) *Drug Metab Dispos.* **30,** 631-635
28. Nguyen, X., Wang, M. H., Reddy, K. M., Falck, J. R., and Schwartzman, M. L. (1999) *Am.J.Physiol* **276,** R1691-R1700
29. Kikuta, Y., Kusunose, E., and Kusunose, M. (2002) *Prostaglandins Other Lipid Mediat.* **68-69,** 345-362
30. Kikuta, Y., Kusunose, E., Kondo, T., Yamamoto, S., Kinoshita, H., and Kusunose, M. (1994) *FEBS Lett.* **348,** 70-74

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31. Nishimura, M., Yaguti, H., Yoshitsugu, H., Naito, S., and Satoh, T. (2003) *Yakugaku Zasshi* **123,** 369-375
32. Christmas, P., Ursino, S. R., Fox, J. W., and Soberman, R. J. (1999) *J.Biol.Chem.* **274,** 21191-21199
33. Kroetz, D. L. and Xu, F. (2005) *Annu.Rev.Pharmacol.Toxicol.* **45,** 413-438
34. Christmas, P., Jones, J. P., Patten, C. J., Rock, D. A., Zheng, Y., Cheng, S. M., Weber, B. M., Carlesso, N., Scadden, D. T., Rettie, A. E., and Soberman, R. J. (2001) *J.Biol.Chem.* **276,** 38166-38172
35. Korman, S. H., Mandel, H., and Gutman, A. (2000) *J.Inherit.Metab Dis.* **23,** 425-428
36. Chen, L. and Hardwick, J. P. (1993) *Arch.Biochem.Biophys.* **300,** 18-23
37. Kawashima, H., Kusunose, E., Thompson, C. M., and Strobel, H. W. (1997) *Arch.Biochem.Biophys.* **347,** 148-154
38. Zhang, X., Chen, L., and Hardwick, J. P. (2000) *Arch.Biochem.Biophys.* **378,** 364-376
39. Kundu, R. K., Getz, G. S., and Tonsgard, J. H. (1993) *J.Lipid Res.* **34,** 1187-1199
40. Kelson, T. L., Secor, M., Jr., and Rizzo, W. B. (1997) *Biochim.Biophys.Acta* **1335,** 99-110
41. Mitz, M. A. and Heinrikson, R. L. (1961) *Biochim.Biophys.Acta* **46,** 45-50
42. Allali-Hassani, A., Peralba, J. M., Martras, S., Farres, J., and Pares, X. (1998) *FEBS Lett.* **426,** 362-366
43. Collins, X. H., Harmon, S. D., Kaduce, T. L., Berst, K. B., Fang, X., Moore, S. A., Raju, T. V., Falck, J. R., Weintraub, N. L., Duester, G., Plapp, B. V., and Spector, A. A. (2005) *J.Biol.Chem.* **280,** 33157-33164

FOOTNOTES

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[1]The abbreviations used are: VLCFA, very long-chain fatty acid; X-ALD, X-linked adrenoleukodystrophy; ALDP, adrenoleukodystrophy protein; ALDR, adrenoleukodystrophy-related protein; PDB, peroxisomal biogenesis disorder; ω-hydroxy-C26:0, 26-hydroxy-hexacosanoic acid; C26:0-DCA, hexacosanedioic acid; DDC, diethyldithiocarbamate; 17-ODYA, 17-octadecynoic acid; CYP, cytochrome P450; PPAR, peroxisome proliferator activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor.

FIGURE LEGENDS

Fig. 1 – Formation of ω-hydroxy-C26:0 and the dicarboxylic acid of C26:0 in human liver microsomes as a function of (A) time, (B) pH and (C) protein. Human liver microsomes (50 µg/ ml) were incubated in a buffered medium containing 100 mM glycine/ 100 mM HEPES pH 8.4, 1 mM NADPH and α-cyclodextrin. Reactions were carried out at 37°C. After termination, the reaction products were analyzed as described in 'Experimental Procedures'. Symbols used: (■) Total product formation, (●) ω-hydroxy-C26:0 and (♦) C26:0-DCA.

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Fig. 2 – Hydroxylation of (A) C22:0, (B) C24:0 and (C) C26:0 by human liver microsomes with the standard VLCFA hydroxylation assay as described in 'Experimental Procedures'. Reactions were carried out at 37°C and terminated after 10 min. All data shown represent the means of two independent experiments. Symbols used: (●) ω-hydroxy fatty acid and (♦) dicarboxylic acid.

Fig. 3 – GC-MS chromatogram (single-ion monitoring mode) of the products from C26:0 hydroxylation in human liver microsomes. Spectrum analysis was performed as described in experimental procedures. The two peaks labeled I and II correspond to the $(M-15)^+$ with m/z 541 of the hydroxylated C26:0 metabolites. Peak III corresponds to the $(M-15)^+$ of the dicarboxylic acid of C26:0 (m/z 555).

Fig. 4 – Characterization of the C26:0 ω-oxidation intermediates from the peaks in Fig. 3. Based on the fragmentation spectra, the trimethylsilyl (TMS) derivative was identified as A) peak II, ω-hydroxy-C26:0; B) peak I, (ω-1)-hydroxy-C26:0 and C) peak III, C26:0-DCA.

Fig. 5 – Effect of several P450 isoform specific inhibitors on the hydroxylation of C26:0 by human liver microsomes. Microsomal protein was pre-incubated in the standard reaction mixture in the presence of inhibitor for 10 min. Subsequently, reactions were initiated by addition of the substrate and were allowed to proceed for 30 min. The data represents the relative inhibition of C26:0 hydroxylation as compared to the activity observed in the absence of inhibitors. The results are the mean of two independent experiments, which did not vary by more than 10%. The color of the bars represents the final inhibitor concentration: (white) 1 μM, (grey) 10 μM and (black) 100 μM. Key: SP, sulfaphenazole; QD, quinidine; KET, ketoconazole; TA, troleandomycin, FF, furafylline; DDC, diethyldithiocarbamate; TMP, trimethoprim; 17-ODYA, 17-octadecynoic acid.

Fig. 6 – Hydroxylation activity of several human recombinant P450 isoforms towards C26:0. Recombinant P450 protein (5 pmol) was incubated for 30 min in the standard VLCFA hydroxylation reaction mixture with C26:0, followed by determination of both ω-hydroxy-C26:0 and C26:0-DCA.

Fig. 7 – Hydroxylation of VLCFAs by human recombinant CYP4F2 at different substrate concentrations. Reactions were initiated by addition of the fatty acid, (A) C22:0, (B) C24:0 and (C) C26:0, for 10 min at 37ºC. The results are the mean of two independent experiments. Symbols used: (●) ω-hydroxy fatty acid and (♦) dicarboxylic acid.

Fig. 8 – ω-Oxidation of VLCFAs by human recombinant CYP4F3B at different substrate concentrations. Hydroxylation of (A) C22:0, (B) C24:0 and (C) C26:0 by human recombinant CYP4F3B at different substrate concentrations and reactions were allowed to proceed for 10 min. All data shown represent the means of two independent experiments. Symbols used: (●) ω-hydroxy fatty acid and (♦) dicarboxylic acid.

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Table 1

Kinetic parameters derived from the experimental data in Figure 2 of VLCFA hydroxylation by human liver microsomes. Values were calculated using a dual-enzyme Michaelis-Menten model as described in "Experimental Procedures". The ω versus (ω-1) hydroxylation ratio was determined by GC-MS analysis as described.

	High affinity			Low affinity			
	K_{m1} μM	V_{max1} nmol·min^{-1}·mg^{-1}	V_{max1}/K_{m1} 10^{-2}	K_{m2} μM	V_{max2} nmol·min^{-1}·mg^{-1}	V_{max2}/K_{m2} 10^{-2}	ratio ω: (ω-1)
C22:0	5.8	0.8	14	402	12.7	3.2	53: 1
C24:0	6.7	0.5	7.5	315	15.9	5.0	61: 1
C26:0	0.1	0.1	110	210	7.3	2.2	32: 1

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Table 2

Kinetic parameters for VLCFA hydroxylation by CYP4F2 and CYP 4F3B. The values were obtained from the experimental data in Fig. 7 and 8 using a two-substrate binding site model as described in experimental procedures. The ω: (ω-1) hydroxylation ratio was determined by GC-MS analysis as described.

	High affinity			Low affinity			
	K_{m1} μM	V_{max1} pmol·min^{-1}·pmol P450^{-1}	V_{max1}/K_{m1}	K_{m2} μM	V_{max2} pmol·min^{-1}·pmol P450^{-1}	V_{max2}/K_{m2}	ratio ω: (ω-1)
CYP4F2							
C22:0	0.5	1.6	3.2	255	67	0.3	42: 1
C24:0	1.1	1.6	1.5	222	50	0.2	52: 1
C26:0	1.9	0.9	0.5	367	11	<0.1	17: 1
CYP4F3B							
C22:0	1.6	5.0	3.1	267	93	0.3	74: 1
C24:0	3.8	9.8	2.6	330	112	0.3	57: 1
C26:0	1.3	2.2	1.7	$7.2 \cdot 10^6$	$4.9 \cdot 10^5$	<0.1	21: 1

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Figure 1

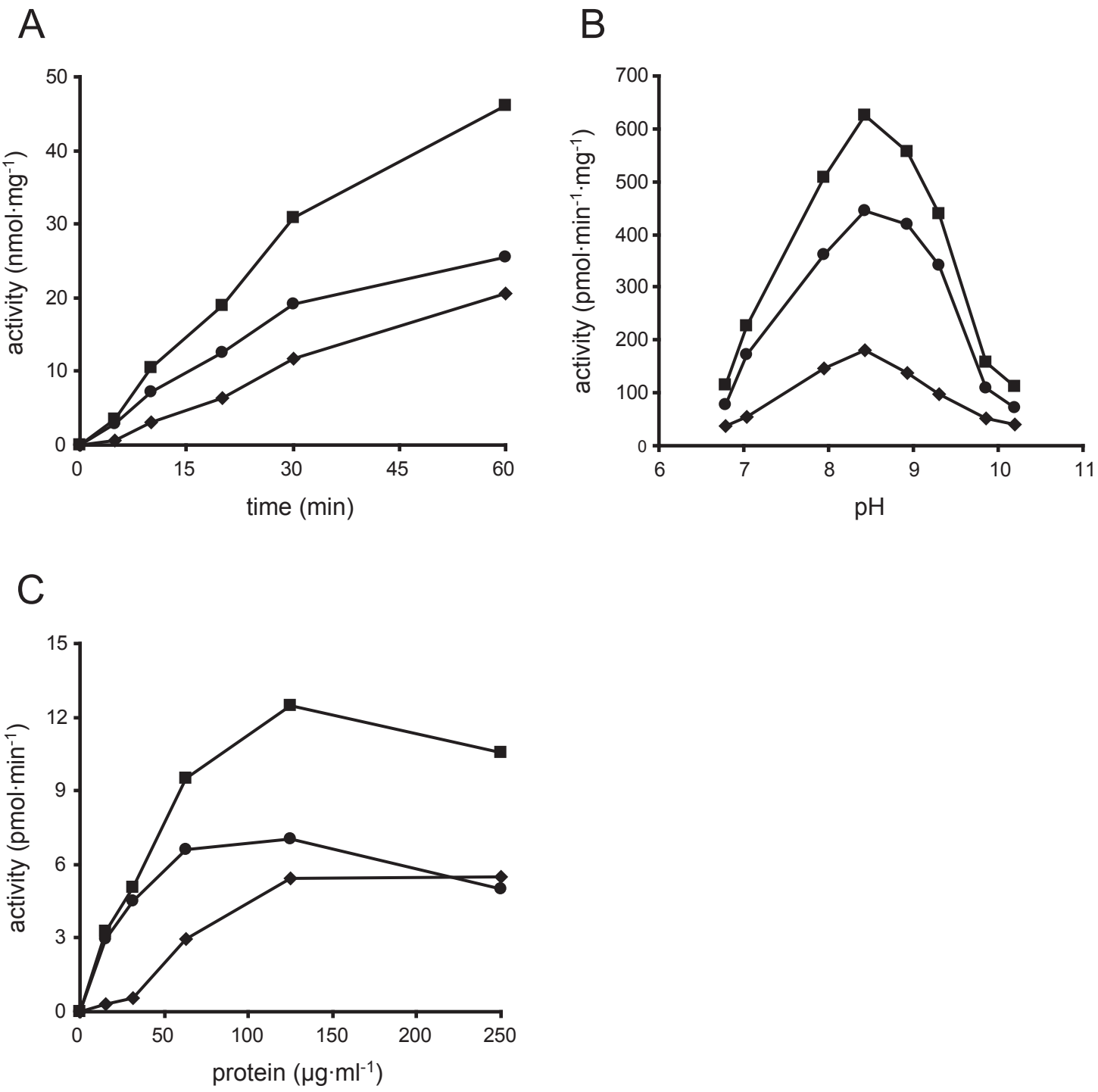

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Figure 2

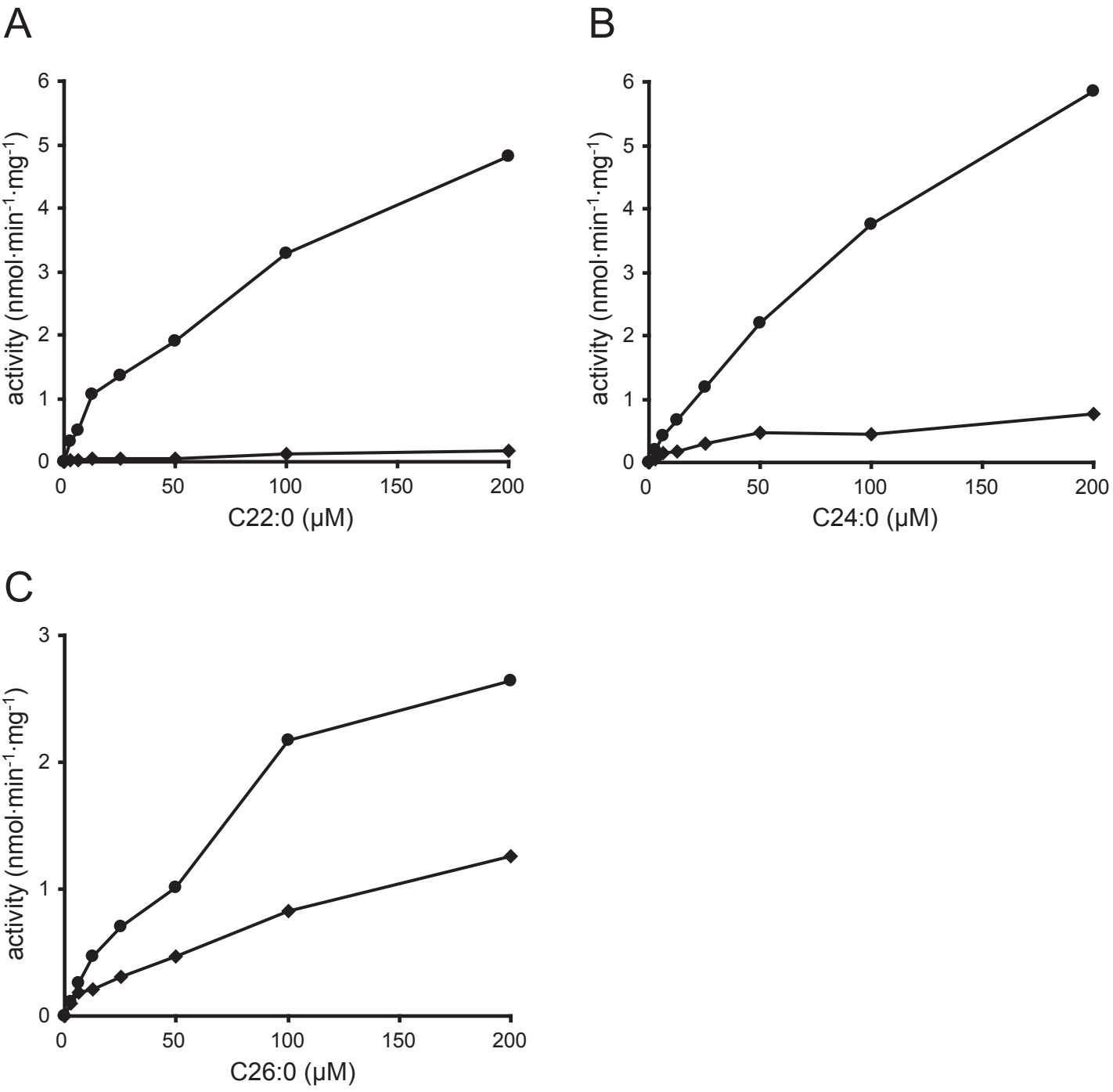

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Figure 3

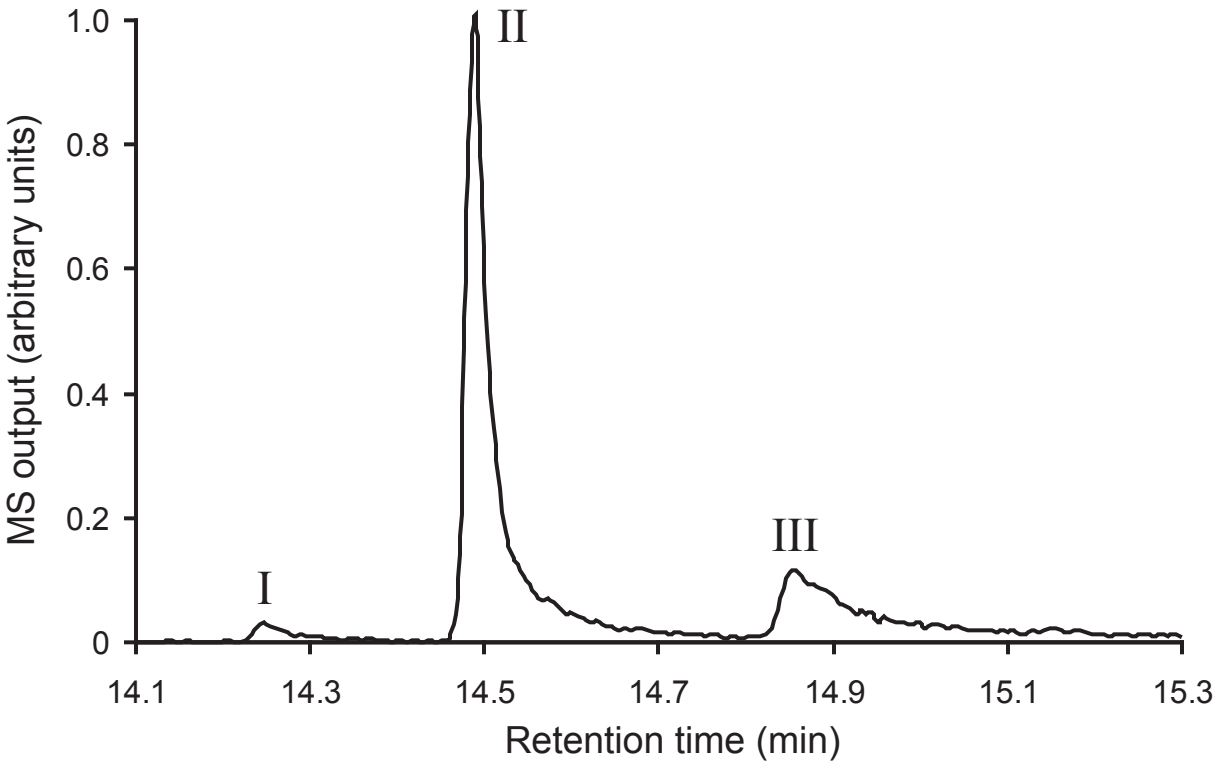

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Figure 4

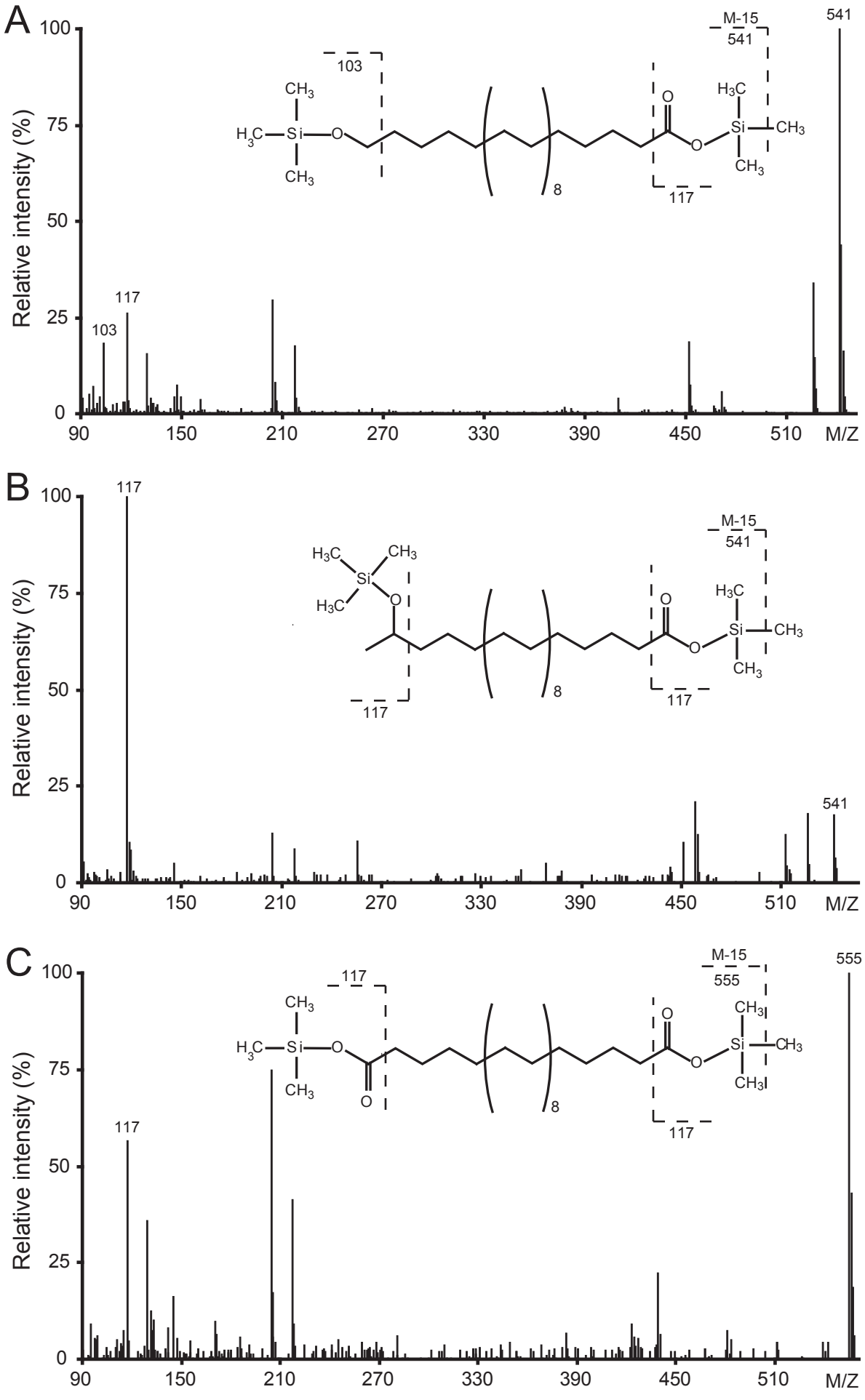

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Figure 5

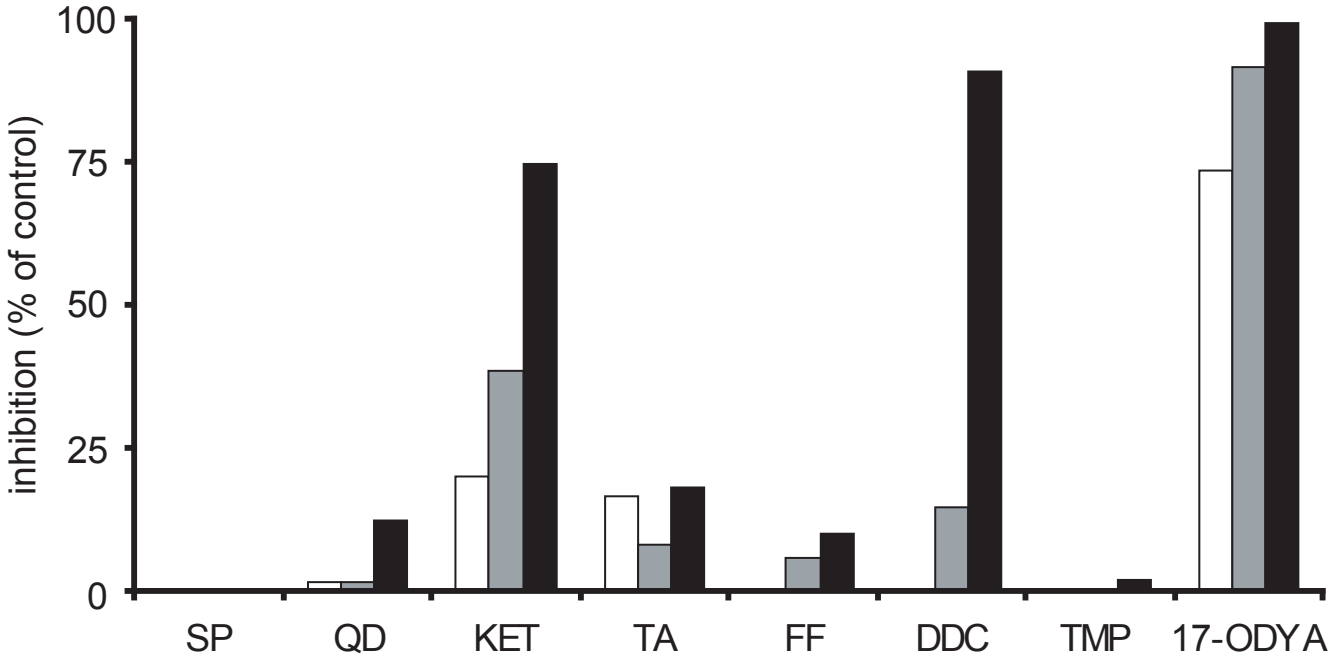

100
75
50
25
0
inhibition (% of control)
SP
QD
KET
TA
FF
DDC
TMP
17-ODYA

Figure 6

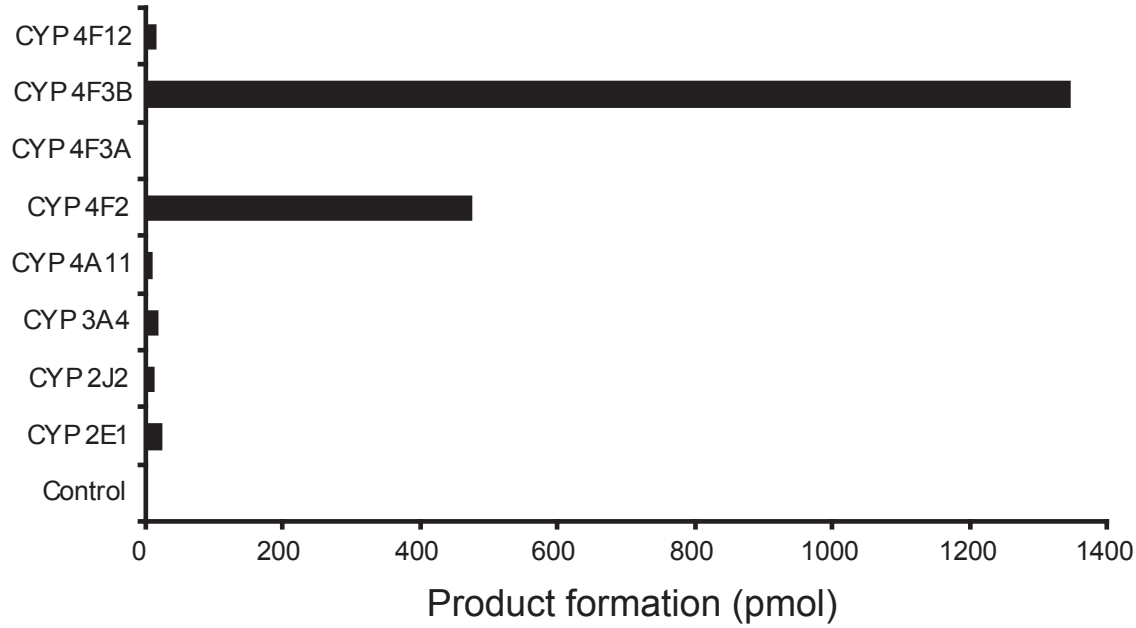

CYP 4F12
CYP 4F3B
CYP 4F3A
CYP 4F2
CYP 4A 11
CYP 3A4
CYP 2J2
CYP 2E1
Control
0
200
400
600
800
1000
1200
1400
Product formation (pmol)

Figure 7

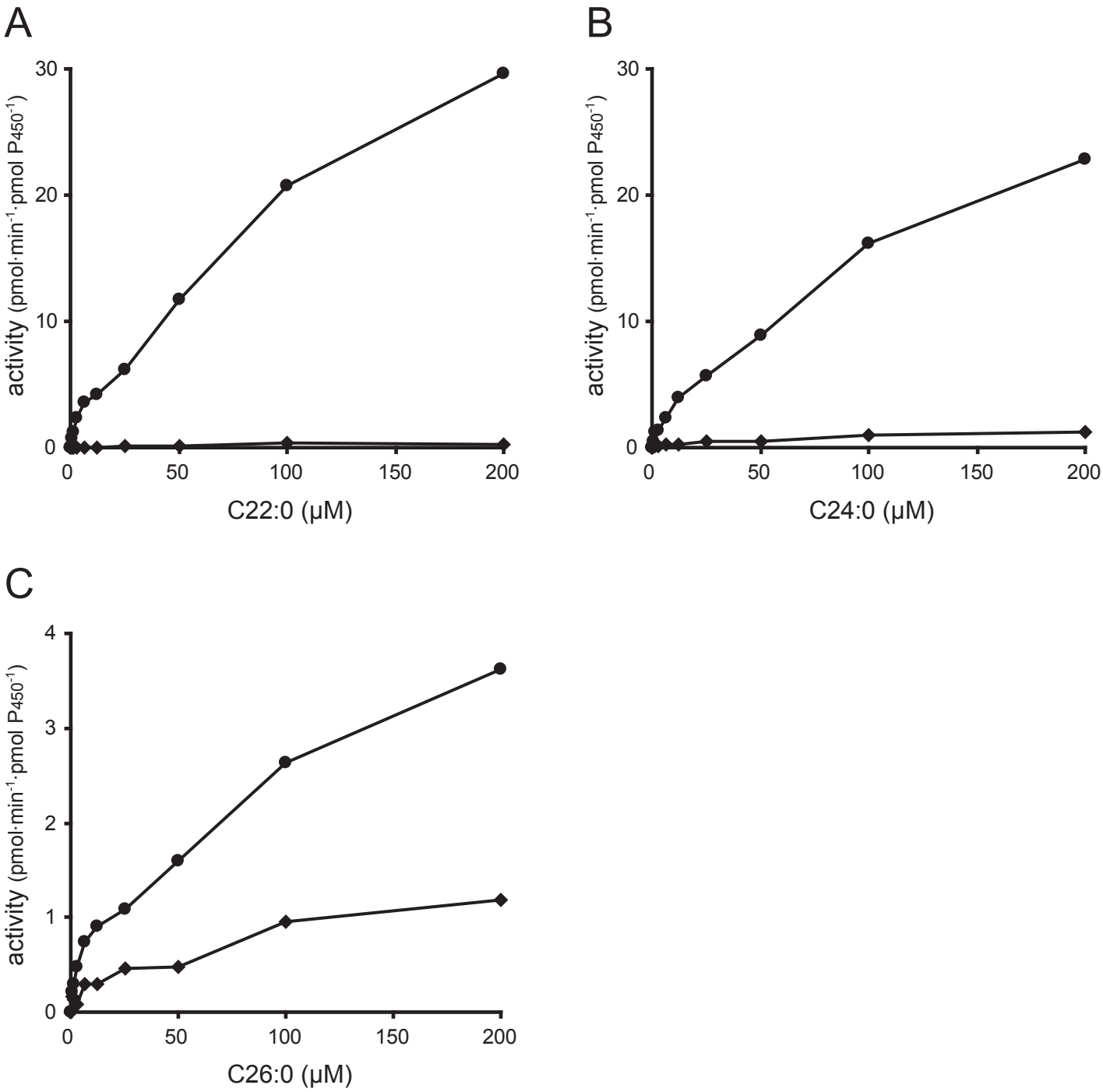
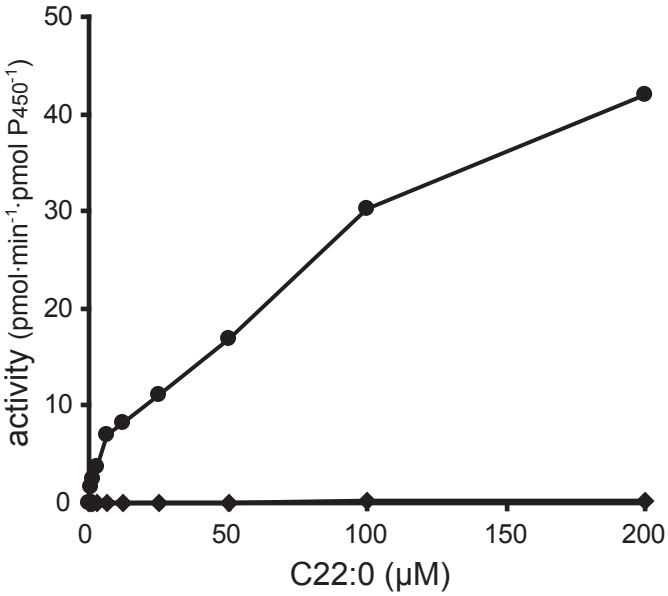
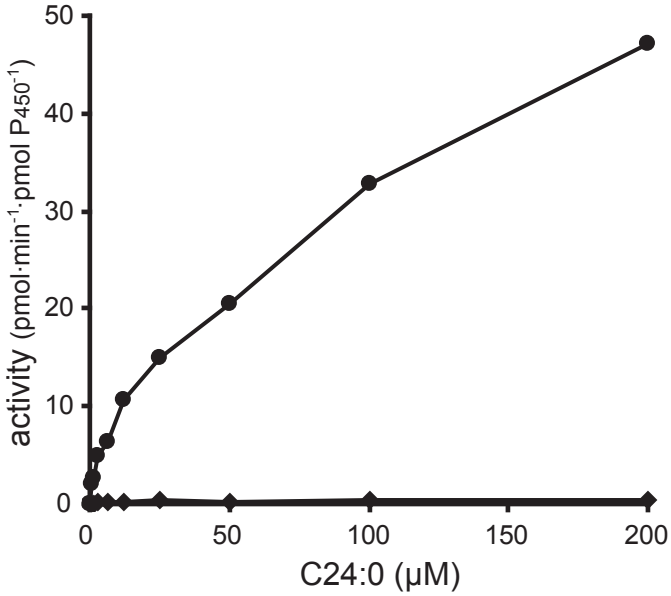

A
B
C
activity (pmol·min-1·pmol P450-1)
C22:0 (μM)
C24:0 (μM)
C26:0 (μM)
0
10
20
30
50
100
150
200
1
2
3
4

Figure 8

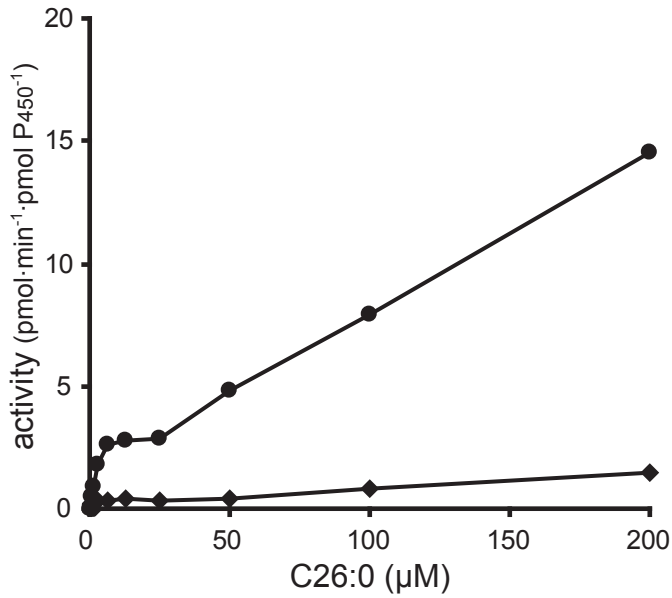
A


activity (pmol·min-1·pmol P450-1)
50
40
30
20
10
0
0
50
100
150
200
C22:0 (μM)

B


activity (pmol·min-1·pmol P450-1)
50
40
30
20
10
0
0
50
100
150
200
C24:0 (μM)

C


activity (pmol·min-1·pmol P450-1)
20
15
10
5
0
0
50
100
150
200
C26:0 (μM)

Omega-oxidation of very long-chain fatty acids in human liver microsomes: Implications for X-linked adrenoleukodystrophy

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