Title: MEANS AND METHODS FOR COUNTERACTING FATTY ACID ACCUMULATION

Abstract: The invention provides a method for at least in part countering a disease involving accumulation of a fatty acid, the method comprising administering a compound which is capable of inducing and/or upregulating omega-oxidation of said fatty acid, or whose metabolite is capable of inducing and/or upregulating omega-oxidation of said fatty acid, to a subject suffering from, or at risk of suffering from, said disease.
Title: Means and methods for counteracting fatty acid accumulation

The invention relates to the field of medicine. More specifically the invention relates to diseases involving accumulation of fatty acids.

Fatty acids are an important source of energy. In an organism fatty acids are constantly broken down and converted into other metabolites. These degradation processes involve the action of a wide variety of enzymes. Most linear fatty acids are degraded by a beta-oxidation pathway in peroxisomes or mitochondria. During beta-oxidation, a fatty acid is shortened by two carbon atoms at the carboxyl end to produce its n-2 analogue.

In general, branched chain fatty acids are also degraded by a beta-oxidation pathway in peroxisomes and mitochondria. However, this is only true for 2-methyl branched chain fatty acids. 3-Methyl branched fatty acids cannot be degraded by a beta-oxidation pathway because the methyl group at the third carbon atom blocks beta-oxidation. Degradation of 3-methyl branched fatty acids therefore proceeds primarily via alpha-oxidation. During alpha-oxidation, a fatty acid is shortened by one carbon atom at the carboxyl end to produce its n-1 analogue. The resulting fatty acid has a 2-methyl group and can be further degraded by beta-oxidation.

A defect in a fatty acid degradation pathway often results in accumulation of a fatty acid or a metabolite thereof. A defective pathway is for instance caused by the absence of an (active) enzyme involved in the degradation of a fatty acid or a metabolite thereof. Other causes of defective fatty acid degradation pathways for instance comprise the absence of an (active) protein involved in the transportation of a fatty acid or a metabolite thereof to a compartment such as a mitochondrion or a peroxisome where degradation should take place. In such a case, a fatty acid or metabolite is not
capable of reaching the enzyme(s) that should degrade it. As a result, accumulation of at least one fatty acid or metabolite takes place.

Accumulation of fatty acids or metabolites thereof over time is mostly harmful for an organism. A wide variety of disorders is known involving the accumulation of one or several fatty acids or metabolites thereof. Examples of such disorders are diabetes mellitus, peroxisomal fatty acid oxidation deficiencies and mitochondrial fatty acid oxidation deficiencies.

Progression of disorders involving the accumulation of fatty acids is often prevented by avoiding the intake of products that comprise, or are converted into, fatty acids that cannot be degraded. Currently it is not possible to counteract fatty acid accumulation disorders by administration of a missing protein to a patient suffering from such disorder. Although other accumulation diseases like Gaucher and Fabry disease are treated by administration of a missing enzyme (also called enzyme replacement therapy), this is not yet possible for fatty acid accumulation diseases.

Reasons for this are that the required protein, such as an enzyme capable of catalysing alpha or beta oxidation of a fatty acid, is not always available, or that a protein does not arrive at the right location within an organism. For instance, administered enzymes are often transported into lysosomes, where they are destroyed.

It is an object of the present invention to provide an alternative method for at least in part counteracting a disorder involving the accumulation of at least one fatty acid.

The invention provides a method for at least in part counteracting a disease involving accumulation of at least one fatty acid, the method comprising administering a compound capable of inducing and/or upregulating omega-oxidation of said fatty acid to a subject suffering from, or
at risk of suffering from, said disease. In one embodiment a compound whose metabolite is capable of inducing and/or upregulating omega-oxidation of said fatty acid is administered to said subject.

According to the present invention, instead of administering an enzyme capable of catalysing alpha-oxidation or beta-oxidation of a fatty acid, it is possible to use an alternative pathway in order to degrade fatty acids. In a method of the invention degradation of a fatty acid via omega-oxidation is induced and/or enhanced. The resulting metabolite is subsequently further degraded. As a result, accumulation of a fatty acid is at least in part diminished. A method of the invention is thus suitable for preventing progression of a disease involving fatty acid accumulation. Moreover, it has become possible to degrade fatty acids which are already accumulated within an organism. Hence, a method of the invention is also suitable for at least in part diminishing the amount of fatty acid that has been built up.

In an omega-oxidation pathway the carbon atom at the omega-end of a fatty acid is hydroxylated by a member of the cytochrome P450 enzyme family. This hydroxylated fatty acid is then converted into an aldehyde by an alcohol dehydrogenase, and subsequently this aldehyde is converted into a carboxyl group by an aldehyde dehydrogenase. As a consequence, the final product of the pathway is a dicarboxylic fatty acid, which can be degraded further within an organism by other pathways, for instance by beta-oxidation from the omega-end.

Cytochrome P450 enzymes naturally occur within organisms. The cytochrome P450 superfamily is highly diverse. Hundreds of P450 sequences are known. They are abundantly present within an organism: most cells comprise one or several cytP450 enzymes. Human individuals possess more than 50 different cytochrome P450 enzymes which are involved in a variety of functions such as drug metabolism, blood hemostasis, cholesterol biosynthesis and steroidogenesis. Although cytochrome P450 enzymes are
capable of catalysing omega-oxidation of fatty acids, they do not naturally
catalyse this reaction to a sufficient extent to compensate for a failing fatty
acid degradation pathway. According to the invention it is however possible
to induce and/or enhance an omega-oxidation capacity of at least one
cytochrome P450 enzyme such that a disorder involving accumulation of at
least one fatty acid is at least in part counteracted. Most cytochrome P450
enzymes are present in the endoplasmic reticulum. They are capable of
catalysing fatty acids that are present in the cytosol. Hence, the amount
and/or omega-oxidation capacity of at least one cytochrome P450 enzyme
which is present in the endoplasmic reticulum is preferably induced and/or
enhanced in order to degrade a fatty acid which is present in the cytosol.
Some cytochrome P450 enzymes are present within mitochondria. The
amount and/or omega-oxidation capacity of at least one of these P450
enzymes is therefore preferably induced and/or enhanced in order to degrade
a fatty acid which is present in mitochondria. As used herein, a cytochrome
P450 enzyme is also referred to as a cytP450.

By at least in part counteracting a disease involving accumulation of a
fatty acid is meant herein that a method is performed which results in a
smaller amount of accumulated fatty acid in an organism as compared to the
amount of accumulated fatty acid that would have been present in said
organism if said method had not been performed.

A compound capable of inducing and/or upregulating omega-oxidation
of a fatty acid is defined herein as:

- a compound whose presence in an organism results in an enhanced level of
  omega-oxidation of a given fatty acid as compared to the level of omega-
  oxidation of said fatty acid that would have been present in said organism if
  said compound had not been present, or
- a compound which is converted \emph{in vivo} into at least one metabolite whose
  presence in an organism results in an enhanced level of omega-oxidation of a
given fatty acid as compared to the level of omega-oxidation of said fatty acid that would have been present in said organism if said metabolite had not been present.

A metabolite of a compound is defined as a molecule which is formed when said compound is processed *in vivo*. After administration of a compound such as for instance a prodrug to an animal, said compound is sometimes altered within said animal. Said compound is for instance cleaved. As another example said compound, or a metabolite thereof, is modified by conjugation with an endogenous molecule such as for instance glucuronic acid, glutathione and/or sulfate. A metabolite resulting from such modification may subsequently be cleaved, and/or a cleavage product may subsequently be modified. Any product resulting from *in vivo* processing of a compound is called herein a metabolite of said compound. If said metabolite is capable of inducing and/or upregulating omega-oxidation of a fatty acid, it is suitable for a method of the present invention. In that case, it is possible to administer said compound and/or at least one suitable metabolite. A compound capable of inducing and/or upregulating omega-oxidation of a fatty acid therefore encompasses compounds that are converted *in vivo* into at least one metabolite which is capable of inducing and/or upregulating omega-oxidation of a fatty acid.

Induction and/or upregulation of omega-oxidation of a fatty acid results in less accumulation of said fatty acid. A compound which is capable of inducing and/or upregulating omega-oxidation of a fatty acid, or whose metabolite is capable of inducing and/or upregulating omega-oxidation of a fatty acid, is therefore suitable for at least in part counteracting a disease involving accumulation of said fatty acid. The invention therefore provides a use of a compound which is capable of upregulating omega-oxidation of a fatty acid, or whose metabolite is capable of inducing and/or upregulating
omega-oxidation of a fatty acid, for the manufacture of a medicament for at least in part treating a disease involving accumulation of said fatty acid.

In a preferred embodiment a method or a use according to the invention is provided wherein said disease comprises diabetes mellitus. In another preferred embodiment said disease comprises a peroxisomal and/or mitochondrial fatty acid oxidation deficiency like, but not restricted to, carnitine palmitoyl transferase 1 and 2 deficiency, very-long-chain acyl-CoA dehydrogenase deficiency, medium-chain acyl-CoA dehydrogenase deficiency, long-chain 3-hydroxy acyl-CoA dehydrogenase deficiency, mitochondrial trifunctional protein deficiency, Refsum disease and/or X-linked adrenoleukodystrophy. In a most preferred embodiment said disease comprises diabetes mellitus, a mitochondrial fatty acid oxidation deficiency, Refsum disease and/or X-linked adrenoleukodystrophy.

Omega-oxidation of a given fatty acid is induced and/or upregulated in various ways. In one embodiment omega-oxidation is induced and/or upregulated by administering a cytochrome P450 enzyme or a functional part, derivative and/or analogue thereof. As stated before, omega-oxidation is catalysed by at least one cytochrome P450 enzyme. Hence, omega-oxidation of a given fatty acid is induced and/or enhanced by administration of at least one cytP450 capable of catalysing omega-oxidation of said given fatty acid, or a functional part, derivative and/or analogue said cytP450. Catalysing omega-oxidation of a fatty acid means that at least one step of an omega-oxidation reaction is catalysed. Preferably, hydroxylation of the carbon atom at the omega-end of a fatty acid is catalysed. The terms "omega-oxidation catalysing property", "omega-hydroxylation capacity" and "fatty acid hydroxylation capacity" are used herein interchangeably. In a preferred embodiment a cytochrome P450 enzyme derived from the same species as the subject to be treated is used. For instance, in order to treat a human individual a human cytochrome P450 or a functional part, derivative and/or analogue thereof is
preferably used. Such human cytochrome P450 molecule is for instance produced by an expression system that has been provided with a nucleic acid sequence encoding a human cytochrome P450 enzyme or a functional part, derivative and/or analogue thereof.

In another embodiment omega-oxidation is induced and/or upregulated by administration of a nucleic acid sequence encoding a cytochrome P450 enzyme or a functional part, derivative and/or analogue of said enzyme. According to this embodiment a subject suffering from, or at risk of suffering from, a disease involving accumulation of a given fatty acid is provided with a nucleic acid sequence encoding a cytP450 capable of catalysing omega-oxidation of said fatty acid. Uses of nucleic acid sequences for administration of a proteinaceous molecule are well known in the art and need no further description here. Gene delivery vehicles for introducing a nucleic acid sequence into an organism are well known in the art. For instance, a (retro)viral vector is used. Said nucleic acid is either constitutively or inducibly expressed. In one embodiment expression of said nucleic acid is controlled by an inducible promoter. In one embodiment said nucleic acid sequence is only significantly expressed in one, or several, kind(s) of tissue(s). For instance said nucleic acid sequence is operably linked to a promoter which is specific for one, or several, kind(s) of tissue(s).

It is of course also possible to use other kinds of nucleic acid structures such as, but not limited to, a DNA/RNA helix, peptide nucleic acid (PNA), locked nucleic acid (LNA) and/or a ribozyme.

A functional part of a cytochrome P450 enzyme is defined as a part of said enzyme which has at least one same enzymatic property in kind, not necessarily in amount. By an enzymatic property is preferably meant the capability to catalyse omega-oxidation of a given fatty acid. A functional derivative of a cytP450 is defined as a cytP450 which has been altered such that at least one enzymatic property of said molecule is essentially the same.
in kind, not necessarily in amount. A derivative can be provided in many ways, for instance through conservative amino acid substitution. Conservative amino acid substitution involves replacement of an amino acid with another amino acid with generally similar properties (size, hydrophobicity, etc), such that the overall functioning is not seriously affected.

A person skilled in the art is well able to generate analogous compounds of a cytP450. This can for instance be done through screening of a peptide library. Such an analogue has essentially the same immunogenic properties of said protein in kind, not necessarily in amount. An analogue of a cytP450 for instance comprises a fusion protein.

A method or use of the invention preferably involves upregulation of the availability, preferably the amount and/or omega-hydroxylation capacity, of at least one endogenous cytochrome P450 enzyme. An advantage of upregulating an organism's own pathway is that possible adverse side effects caused by exogenous enzymes are at least in part avoided.

In one preferred embodiment omega-oxidation of a given fatty acid is induced and/or enhanced by a compound capable of increasing the availability of at least one cytochrome P450 enzyme capable of catalysing omega-oxidation of said fatty acid. The availability of cytP450 for instance comprises the amount of cytP450 that is capable of catalysing omega-oxidation, and/or the omega-hydroxylation capacity of a cytP450 enzyme. In a preferred embodiment a compound capable of increasing the amount of at least one cytochrome P450 enzyme is used. In other embodiments the availability of a cytochrome P450 enzyme is increased by removing a compound bound to said cytP450, by transport of said cytP450 to a desired site, et cetera. Additionally, or alternatively, a compound is used that is capable of enhancing the omega-hydroxylation capacity of a cytP450. By enhancing the omega-hydroxylation capacity of a cytochrome P450 enzyme is meant that at least one parameter
of an omega-hydroxylation reaction catalysed by said cytochrome P450 enzyme is improved. For instance, in one embodiment a compound is used which enhances the affinity with which cytP450 is capable of binding a given fatty acid. In another embodiment a compound is used which enhances the reaction rate of omega-hydroxylation catalysed by a cytP450. Various other ways of enhancing the omega-hydroxylation capacity of a cytochrome P450 enzyme are known in the art.

An amount of a cytochrome P450 enzyme is upregulated in various ways. In one embodiment, degradation of a cytochrome P450 enzyme is at least in part inhibited. This results in a higher amount of cytochrome P450 in a cell. In one preferred embodiment an amount of a cytP450 is upregulated by inducing and/or enhancing expression of said cytP450. This is for instance performed by administration of a compound capable of inducing or enhancing an inducible cytP450-specific promoter. Many cytP450 enzyme genes are under control of at least one inducible promoter. In that case it is possible to induce and/or enhance at least one inducible cytP450-specific promoter. This is for instance performed by administration of one or more compounds capable of inducing and/or enhancing at least one promoter. In one preferred embodiment at least two inducible cytP450-specific promoters are induced or enhanced. In one embodiment, a compound capable of inducing and/or enhancing a first cytP450-specific promoter and another compound capable of inducing and/or enhancing a second cytP450-specific promoter is used. Alternatively, or additionally, a compound capable of inducing at least two cytP450-specific promoters is used. In one embodiment one or several compounds capable of inducing and/or enhancing promoters of at least two different cytP450 enzymes are used.

In one embodiment said promoter is directly induced or enhanced by said compound. For instance, a compound capable of directly binding said promoter, thereby inducing expression of cytP450, is suitable. Additionally, or
alternatively, said promoter is indirectly induced or enhanced. In one preferred embodiment a compound is used that is capable of binding another compound or complex, the resulting complex being capable of binding and/or inducing said cytP450 promoter. In one embodiment a compound is used that is capable of binding a cytP450 enhancer or silencer.

In a preferred embodiment expression of cytP450 is induced and/or enhanced by a ligand of a member of the nuclear hormone family. The nuclear hormone family is well known in the art and represents a group of transcription factors with a similar structure which are activated by different ligands. PPAR-alpha, LXR, FXR, PXR and CAR are preferred examples of members of the nuclear hormone family. Said nuclear hormone family member preferably comprises a compound which is naturally bound to retinoic X receptor (RXR), since RXR-nuclear hormone family – ligand complexes are particularly suitable for inducing and/or enhancing cytP450 expression by binding to a response element in the promoter region of a cytP450 gene. Preferably a ligand of a peroxisome proliferator-activated receptor alpha (PPAR-alpha), liver X receptor (LXR), farnesoid X receptor (FXR), pregnane X receptor (PXR) or constitutively active receptor (CAR) is used. Suitable ligands of nuclear hormone family members are listed in Table 1 (derived from Honkakoski et al, 2000).

According to one embodiment, binding of a ligand to a member of a nuclear hormone family bound to RXR is followed by binding of the resulting RXR - nuclear hormone family member- ligand complex to an inducible promoter of a cytP450. Said binding results in (enhanced) expression of cytP450. One embodiment therefore provides a method or use according to the invention wherein said compound capable of inducing and/or upregulating omega oxidation comprises a compound capable of directly or indirectly inducing or enhancing an inducible promoter of cytP450. Said compound preferably comprises a ligand of the nuclear hormone family, most preferably a ligand of PPAR-alpha, LXR, FXR, PXR and/or CAR.
A preferred example of suitable ligands of PPAR-alpha are fibrates. Fibrates are a class of amphipathic carboxylic acids. Fibrates are already used in the art for a range of metabolic disorders, mainly in order to reduce cholesterol and triglyceride levels in the blood. A use of a fibrate for inducing and/or upregulating omega-oxidation of a fatty acid is not shown nor suggested before. The use of a fibrate in a method of the present invention is preferred because fibrates are already known to be suitable for use as medicaments without - or with acceptable - side effects. In a preferred embodiment bezafibrate, fenofibrate, gemfibrozil, and/or ciprofibrate is used. These fibrates are already used as a therapeutic agent in human beings, although for entirely different purposes. These fibrates are thus already known to be suitable for therapeutic use in humans, without - or with acceptable - side effects. In another preferred embodiment rifampicin and/or phenytoin is used in order to at least partly counteract a disease involving accumulation of fatty acid. Like fibrates, these compounds are known therapeutics, albeit for different diseases. Administration of these compounds to human beings has already been approved. A use of rifampicin and/or phenytoin for inducing and/or upregulating omega-oxidation of a fatty acid is neither shown nor suggested before.

The invention thus provides a method or use of the invention wherein said compound capable of inducing and/or upregulating omega-oxidation of fatty acid comprises a fibrate, rifampicin and/or phenytoin. In a preferred embodiment said fibrate comprises bezafibrate, fenofibrate, gemfibrozil, and/or ciprofibrate. In a further preferred embodiment said compound comprises a compound as listed in Table 1. Of course various other compounds capable of directly or indirectly inducing or enhancing the availability of a cytP450 enzyme are suitable for use in a method of the invention.

The art also provides various methods for enhancing the omega-hydroxylation capacity of a cytochrome P450 enzyme. For instance, a
compound capable of enhancing enzymatic activity is administered. Alternatively, or additionally, a compound capable of increasing the availability of a substrate of cytP450 is used. For instance, a compound capable of increasing solubilization of fatty acid is used. In one embodiment a compound is used which enhances binding of a fatty acid to a cytP450 enzyme.

In one aspect a method or use of the invention is provided wherein said cytochrome P450 enzyme comprises a human cytochrome P450 enzyme. Inducing and/or upregulating the availability of at least one human cytochrome P450 enzyme is particularly suitable for therapeutic treatment of a human individual. Preferably, the availability of endogenous human cytP450 is enhanced.

In one embodiment a method or use of the invention is combined with a conventional treatment method, such as enzyme replacement therapy. Additionally, or alternatively, at least two embodiments of a method of the present invention are performed. For instance, a method comprising upregulating and/or enhancing a cytochrome P450-specific promoter in an individual is combined with administration of cytochrome P450 to said individual. Said at least two embodiments may be performed simultaneously. It is also possible to firstly start with one method of the invention, and subsequently start with another method of the invention. In one embodiment a compound capable of upregulating and/or enhancing a cytochrome P450-specific promoter such as a ligand of a nuclear hormone family member is administered together with (at least one nucleic acid encoding) at least one cytP450. In another embodiment said compound capable of upregulating and/or enhancing a cytochrome P450-specific promoter and said (at least one nucleic acid encoding) at least one cytP450 are administered at different time points.
The invention furthermore provides a use of a non-human animal suffering from, or at risk of suffering from, a disease involving accumulation of a fatty acid for determining whether a compound is capable of inducing and/or upregulating omega-oxidation of said fatty acid. It is for instance tested whether administration of a candidate compound to such animal results in decreased accumulation of fatty acid. Decreased fatty acid accumulation is for instance demonstrated by measuring said fatty acid(s) in plasma and/or tissues taken from treated and non-treated animals using, for instance, gas chromatography and/or tandem mass spectrometry. If accumulation of said fatty acid is decreased, it indicates that said candidate compound is capable of at least in part inducing and/or enhancing omega-oxidation of said fatty acid.

Moreover, such animal is suitable for testing possible negative side effects of a compound which is capable of inducing and/or upregulating omega-oxidation of a fatty acid.

The invention therefore provides a method for determining whether and/or to what extent a compound is capable of inducing and/or upregulating omega-oxidation of a fatty acid, comprising providing a non-human animal suffering from, or at risk of suffering from, a disease involving accumulation of said fatty acid with said fatty acid and with said compound, determining the extent of accumulation of said fatty acid in said animal, and comparing said extent of accumulation with the extent of accumulation of said fatty acid in the same kind of non-human animal which is not, or to a significantly lesser extent, provided with said compound.

In one embodiment said fatty acid comprises C26:0 or phytic acid.
Detailed description

Two non-limiting examples of fatty acid accumulation disorders that are at least in part counteracted with a method or use of the present invention are Refsum disease and X-linked adrenoleukodystrophy.

Adult Refsum Disease (ARD) is an autosomal recessive disorder caused by deficient alpha-oxidation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). In the majority of patients this is due to mutations in the gene encoding phytanoyl-CoA hydroxylase, a peroxisomal enzyme. In a subset of patients mutations in the PEX7 gene have been found. Phytanoyl-CoA hydroxylase catalyzes the first step in the alpha-oxidation pathway of 3-methyl branched-chain fatty acids. These fatty acids require alpha-oxidation for their degradation since the 3-methyl group blocks breakdown by regular beta-oxidation. During alpha-oxidation, phytanic acid is converted into its n-1 analogue pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which can readily be degraded by peroxisomal beta-oxidation.

The deficiency of alpha-oxidation in ARD patients leads to the gradual accumulation of phytanic acid. Elevated phytanic acid in the absence of abnormalities in any of the other peroxisomal parameters including plasma very long chain fatty acids, bile acid intermediates and erythrocyte plasmalogens is suggestive for ARD. Classical symptoms are: progressive retinitis pigmentosa, peripheral neuropathy, anosmia, and cerebellar ataxia. The only treatment available at the moment is a diet low in phytanic acid, which may be preceded by plasmapheresis. This alleviates the phytanic acid accumulation and slows down the progression of the disease.

X-linked adrenoleukodystrophy (X-ALD) is an inherited metabolic disorder involving accumulation of C26:0. People with ALD accumulate high levels of saturated, very long chain fatty acids in their brain and adrenal
cortex because the fatty acids cannot be broken down. X-ALD is clinically heterogeneous with at least 6 subtypes of which childhood cerebral ALD (CCALD) and adrenomyeloneuropathy (AMN) are most frequent occurring in > 80% of patients.

In one embodiment, a method or use of the invention in order to at least in part counteract accumulation of phytanic acid and/or C26:O is provided. According to the present invention, the human cytochrome P450 enzymes CYP4A11, CYP4F2, CYP4F3A and CYP4F3B are capable of catalysing omega-oxidation of phytanic acid. Omega-oxidation of phytanic acid leads to 1,16-phytanedioic acid which is further degraded by beta-oxidation from its omega-end. Hence, increasing the availability of at least one of these human cytochrome P450 enzymes results in at least partial treatment of Refsum disease. In one embodiment Refsum disease is therefore at least in part counteracted by administration of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B or a functional part, derivative and/or analogue thereof. According to the present invention the phytanic acid omega-oxidation catalyzing activity of CYP4F3A is highest (as compared to CYP4A11, CYP4F2 and CYP4F3B) followed by, in descending order, CYP4F3B, CYP4F2 and CYP4A11. Therefore, in one preferred embodiment CYP4F3A, CYP4F3B and/or CYP4F2, or a functional part, derivative and/or analogue thereof, is administered. More preferably, CYP4F3A and/or CYP4F3B, or a functional part, derivative and/or analogue thereof, is administered. Most preferably, CYP4F3A, or a functional part, derivative and/or analogue thereof, is administered. Said enzymes are suitable for use as a medicament. The invention therefore provides CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B for use as a medicament. More preferably, CYP4F3A, CYP4F3B and/or CYP4F2, or a functional part, derivative and/or analogue thereof, is used as a medicament. More preferably, CYP4F3A and/or CYP4F3B, or a functional part, derivative and/or analogue thereof, is used as a medicament. Most
preferably, CYP4F3A, or a functional part, derivative and/or analogue thereof, is used as a medicament. A use of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B or a functional part, derivative and/or analogue thereof for the preparation of a medicament for at least in part treating Refsum disease is therefore also herewith provided. More preferably, CYP4F3A, CYP4F3B and/or CYP4F2, or a functional part, derivative and/or analogue thereof, is used. More preferably, CYP4F3A and/or CYP4F3B, or a functional part, derivative and/or analogue thereof, is used. Most preferably, CYP4F3A, or a functional part, derivative and/or analogue thereof, is used.

In one embodiment a nucleic acid sequence encoding CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B or a functional part, derivative and/or analogue thereof is used as a medicament. Expression of a nucleic acid sequence encoding CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B or a functional part, derivative and/or analogue thereof results in an (enhanced) amount of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, which enzymes are particularly suitable for counteracting Refsum disease. In one embodiment, an exogenous nucleic acid sequence encoding CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B is used. In another embodiment, endogenous CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B is overexpressed. CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B is preferably (over)expressed in the liver because the concentration of other compounds involved in omega-oxidation, such as NADPH: CYP450 oxidoreductase is particularly high in the liver. Expression in the liver is for instance favoured by using a promoter which is particularly active in, and/or specific for, liver cells. CYP4F3A, CYP4F3B and/or CYP4F2 is/are preferably (over)expressed because these enzymes are particularly effective in catalyzing omega-oxidation of phytanic acid. CYP4F3A and/or CYP4F3B are most preferably (over)expressed because these enzymes most effectively catalyse omega-oxidation of phytanic acid as
compared to CYP4F2 and CYP4A11. Most preferably, CYP4F3A is
(over)expressed.

Another embodiment provides a method or use of the invention
5 wherein a compound is used which compound, or its metabolite, is capable of
inducing or upregulating the availability, preferably the amount and/or the
omega-hydroxylation capacity, of CYP4A11, CYP4F2, CYP4F3A and/or
CYP4F3B. Such compound is also suitable for the preparation of a
medicament. A use of a compound which is capable of increasing the amount
and/or the omega-hydroxylation capacity of CYP4A11, CYP4F2, CYP4F3A
and/or CYP4F3B, or whose metabolite is capable of increasing the amount
and/or the omega-hydroxylation capacity of CYP4A11, CYP4F2, CYP4F3A
and/or CYP4F3B, for the preparation of a medicament for at least in part
treating Refsum disease is therefore also herewith provided.

Furthermore, the invention provides a method for at least in part
treating Refsum disease the method comprising administering a compound
which is capable of increasing the amount and/or the omega-hydroxylation
capacity of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, or whose
metabolite is capable of increasing the amount and/or the omega-
hydroxylation capacity of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, to
a subject suffering from, or at risk of suffering from, Refsum disease.

CYP4A11 expression is controlled by PPAR-alpha. PPAR-alpha is
capable of binding retinoic X receptor (RXR) and a PPAR-alpha ligand.
RXR - PPAR-alpha - ligand complexes are, in turn, capable of binding a
25 CYP4A11 promoter, which results in (enhanced) expression of CYP4A11.
Administration of a PPAR-alpha ligand thus results in upregulation of
CYP4A11 and, as a result, upregulation of phytanic acid omega-oxidation.
Suitable ligands of PPAR-alpha are fibrates. Fibrates are therefore
particularly suitable for upregulating CYP4A11 expression, resulting in
enhanced omega-oxidation of phytanic acid. As a result, Refsum disease is
counteracted. The invention therefore provides a use of a fibrate for inducing and/or enhancing omega oxidation of phytanic acid. The invention also provides a method for counteracting Refsum disease comprising administering a fibrate to a subject suffering from, or at risk of suffering from, Refsum disease. A use of a fibrate for the preparation of a medicament for counteracting Refsum disease is also herewith provided. In a particularly preferred embodiment fenofibrate is used. Phytanic acid omega-oxidation is significantly increased after fenofibrate administration. One preferred embodiment therefore provides a use of fenofibrate in order to induce and/or enhance phytanic acid omega-oxidation. The invention also provides a method for counteracting Refsum disease comprising administering fenofibrate to a subject suffering from, or at risk of suffering from, Refsum disease. A use of fenofibrate for the preparation of a medicament for counteracting Refsum disease is also herewith provided.

According to another aspect of the invention, the human cytochrome P450 enzymes CYP4F2 and/or CYP4F3B are capable of catalysing omega-oxidation of C26:O. Hence, increasing the availability of CYP4F2 and/or CYP4F3B results in at least partial treatment of X-linked adrenoleukodystrophy. In one embodiment X-linked adrenoleukodystrophy is therefore at least in part counteracted by administration of CYP4F2 and/or CYP4F3B or a functional part, derivative and/or analogue thereof. According to the present invention the C26:O omega-oxidation catalyzing activity of CYP4F2 is highest, as compared to CYP4F3B. Therefore, in one preferred embodiment CYP4F2, or a functional part, derivative and/or analogue thereof, is administered. CYP4F2 and/or CYP4F3B, or a functional part, derivative and/or analogue thereof, for use as a medicament is also herewith provided, as well as a use of CYP4F2, CYP4F3B or a functional part, derivative and/or analogue thereof for the preparation of a medicament for at least in part treating X-linked adrenoleukodystrophy. Most preferably,
CYP4F2, or a functional part, derivative and/or analogue thereof, is used as a medicament. CYP4F2, or a functional part, derivative and/or analogue thereof, is most preferably used for the preparation of a medicament for at least in part treating X-linked adrenoleukodystrophy.

In one embodiment a nucleic acid sequence encoding CYP4F2 and/or CYP4F3B, or a functional part, derivative and/or analogue thereof, is used as a medicament. Expression of a nucleic acid sequence encoding CYP4F2 and/or CYP4F3B, or a functional part, derivative and/or analogue thereof, results in an (enhanced) amount of CYP4F2 and/or CYP4F3B, which enzymes are particularly suitable for counteracting X-linked adrenoleukodystrophy. In one embodiment, an exogenous nucleic acid sequence encoding CYP4F2 and/or CYP4F3B is used. In another embodiment, endogenous CYP4F2 and/or CYP4F3B is overexpressed. CYP4F2 and/or CYP4F3B is/are preferably (over)expressed in the liver because the concentration of other compounds involved in omega-oxidation, such as NADPH: CYP450 reductase is particularly high in the liver. Expression in the liver is for instance favoured by using a promoter which is particularly active in, and/or specific for, liver cells. CYP4F2 is most preferably (over)expressed because this enzyme most effectively catalyses omega-oxidation of C26:O, as compared to CYP4F3B.

One embodiment provides a method or use of the invention wherein a compound is used which compound, or whose metabolite, is capable of inducing or upregulating the availability, preferably the amount and/or the omega-hydroxylation capacity, of CYP4F2 and/or CYP4F3B. Such compound is also suitable for the preparation of a medicament. The invention therefore also provides a use of a compound which is capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F2 and/or CYP4F3B, or whose metabolite is capable of increasing the amount and/or the omega-
hydroxylation capacity of CYP4F2 and/or CYP4F3B, for the preparation of a medicament for at least in part treating X-linked adrenoleukodystrophy. A method for at least in part treating X-linked adrenoleukodystrophy the method comprising administering a compound which is capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F2 and/or CYP4F3B, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F2 and/or CYP4F3B, to a subject suffering from, or at risk of suffering from, X-linked adrenoleukodystrophy is also herewith provided.

The invention furthermore provides a kit comprising a compound capable of increasing the amount and/or the omega-hydroxylation capacity of an enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of an enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B. Said kit is particularly suitable for at least in part counteracting Refsum disease and/or X-linked adrenoleukodystrophy. In one embodiment, at least two compounds capable of increasing the amount and/or the omega-hydroxylation capacity of an enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B are used. One embodiment therefore provides a kit comprising a first compound capable of increasing the amount and/or the omega-hydroxylation capacity of a first enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of a first enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, and a second compound capable of increasing the amount and/or the omega-hydroxylation capacity of a second enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, or whose metabolite is capable of increasing the
amount and/or the omega-hydroxylation capacity of a second enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B.

5 Said first and second compounds are preferably different from each other. In one embodiment said first and second enzymes are different enzymes.

10 The invention furthermore provides a kit dedicated to the treatment of X-linked adrenoleukodystrophy. Said kit preferably comprises a compound capable of increasing the availability, preferably the amount and/or the omega-hydroxylation capacity, of CYP4F2 and/or CYP4F3B since the human cytochrome P450 enzymes CYP4F2 and/or CYP4F3B are capable of catalysing omega-oxidation of C26:O, resulting in at least partial treatment of X-linked adrenoleukodystrophy. In one preferred embodiment the availability of CYP4F2 and CYP4F3B is enhanced. One embodiment therefore provides a kit comprising a compound capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F2, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F2, and a compound capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F3B, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F3B. Said two compounds are preferably different from each other.

20 The invention is further explained in the following examples. These examples do not limit the scope of the invention, but merely serve to clarify the invention. Many alternative embodiments can be carried out, which are within the scope of the present invention.
Examples

Example 1

Material and methods

Materials

Phytanic acid and 3-hydroxyheptadecanoic acid were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). NADPH and NAD⁺ were obtained from Roche (Mannheim, Germany). Clotrimazole, ketoconazole, bifonazole and miconazole were purchased from Sigma (St. Louis, MO). Methyl-beta-cyclodextrine was from Fluka (Buchs, Switzerland).

Preparation of rat liver microsomes

Microsomes were isolated from rat livers by differential centrifugation essentially as described Baudhuin et al. (Baudhuin, 1964). To this end, Male Wistar rats fed a standard laboratory diet, were fasted overnight before sacrifice and removal of the liver. The livers were rapidly chilled and washed several times in buffer containing 250 mM sucrose, 0.5 mM EDTA, 2 mM MOPS/KOH (final pH 7.4). Subsequently, the livers were minced and homogenized with a Potter S homogenizer (B. Braun, Germany) with a teflon pestle at 500 rpm (5 strokes), followed by centrifugation of the homogenate for 10 min at 550 × g. The obtained post-nuclear supernatant was subjected to centrifugation at 22,500 × g for 10 min to remove mitochondria and lysosomes. Finally, the microsomal fraction was obtained by centrifugation of the supernatant for 3 h at 32,000 × g. The microsomal pellet fraction was taken up in PBS containing 5 mM DTT and divided into small aliquots, which were stored at -80°C. The microsomes were sonicated 3 times for 10 s at 8
Watt before each experiment. The protein concentration of the microsomal fraction was determined with the method described by Bradford (Bradford, 1976).

Phytic acid omega(-1)-hydroxylase assay

The standard reaction mixture consisted of 100 mM potassium phosphate buffer pH 7.4 and rat liver microsomes (1 mg/ml end concentration) plus phytanic acid dissolved in DMSO (200 μM end concentration, unless indicated otherwise). Reactions were initiated by addition of NADPH at a final concentration of 1 mM. The final reaction volume was 0.2 ml. Reactions were terminated by addition of 0.2 ml 1 M HCl. Subsequently, 1 ml PBS was added followed by addition of 0.1 ml 12.1 M HCl. The internal standard (IS, 10 nmol 3-hydroxyheptadecanoic acid in 20 μl ethanol) was added to this aqueous mixture. The samples were extracted twice with 6 ml ethylacetate-diethylether (1:1 v/v). The organic layer was collected and the solvents evaporated under vacuum using a rotary evaporator at room temperature. The residue was dissolved in 4 ml ethylacetate and further dried with MgSO₄. After spinning down the MgSO₄, the solution was transferred to 4 ml reaction vials and the solvent evaporated under nitrogen. To enable gas chromatography-mass spectrometry (GS/MS) analysis the extracted fatty acids were derivatized to their corresponding trimethylsilyl (TMS) compounds essentially using the procedure described by Chalmers and Lawson (Chalmers, 1982). TMS ester/ether formation was performed with 40 μl N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) and 10 μl pyridine. The vials were sealed with a Teflon-lined screwcap and incubated at 80°C for 1 hour. After the incubation the solution could be directly used for GC-MS analysis.
GC/MS
GC/MS was performed on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 mass-selective detector (Palo Alto, CA). Samples (1 µl) were injected in the splitless mode (Hewlett Packard 7683 injector) and analyzed on a CP-Sil 5 CB low bleed MS column (25 m x 0.30 µm) (Chrompack, Middelburg, The Netherlands). The oven temperature was programmed as follows: 70°C for 2 min, 5°C/min to 120°C, 7°C/min to 260°C, 3.5 min hold at 260°C then 15°C/min to 275, hold for 10 min. The identity of the substrate, the internal standard, and the reaction product(s) was verified by taking mass spectra of the permethylsilylated derivatives in the scanning electron impact mode. The single ion monitoring (SIM) mode was applied for the detection of the respective (M-15)+ ions (m/z 369 and 457; masses of the molecular ions minus one methyl group of the TMS derivatives of phytanic acid and omega(-1)-hydroxyphytanic acid respectively). Analyte quantification was done by integration of the peaks followed by dividing the analyte peak areas with the area of the internal standard (TMS derivative of 3-hydroxyheptadecanoic acid, monitored ion m/z 233).

Results

Hydroxylation of phytanic acid by rat liver microsomes
In order to study the omega-oxidation of phytanic acid, rat liver microsomes were incubated in a phosphate buffered medium containing phytanic acid. When NADPH was added, two products appeared in the chromatogram, one with a retention time of 29.1 min and the other with a retention time of 29.7 min (Fig. 1). The major peak was identified as omega-hydroxyphytanic acid (16-hydroxyphytanic acid) according to its mass spectrum (Fig. 2A). Mass spectral analysis of this peak revealed the presence
of a fragment (m/z = 103) characteristic for omega-hydroxyacids, representing the terminal CH₂OSi(CH₃)₃-moiety. The minor peak corresponds to (omega-1)-hydroxyphytanic acid (15-hydroxyphytanic acid) (Fig. 2B), the product of (omega-1)-hydroxylation. A general characteristic of the TMS-derivatives of the hydroxyfatty acids is cleavage of the molecule adjacent to the hydroxyl group. It was deduced from the mass spectrum in Fig. 2B that the base peak at m/z = 131 represents the (CH₃)₂COSi(CH₃)₃-moiety, in analogy with the mass spectrum of 3-hydroxyisovaleric acid.

Optimization of the hydroxylase assay

The hydroxylation assay was further optimized for the formation of omega-hydroxyphytanic acid. First the influence of methyl-beta-cyclodextrin, added to increase solubilization of the substrate, on the formation of omega-hydroxyphytanic acid was tested. Figure 3A shows that methyl-beta-cyclodextrin has a positive effect on the assay with an optimum concentration of 0.75 mg/ml. Higher methyl-beta-cyclodextrin concentrations had a negative effect on the assay, presumably due to decreased substrate availability. To determine the optimal pH value for our assay a combined buffer system with 50 mM potassium phosphate/50 mM pyrophosphate was used to cover the pH range of 6.6 to 9.1. The result depicted in Fig. 3B shows an optimum pH of 7.6. Accordingly, all subsequent experiments were performed in 0.1 M potassium phosphate at pH 7.6.

Our next aim was to analyze the kinetics of the hydroxylation of phytanic acid under the conditions determined in the previous experiments. We already established that NADPH was an essential component of the reaction mixture (Fig. 1). The NADPH dependency of the reaction was studied in more detail by performing the assay at different NADPH concentrations. For this purpose, we included a NADPH regenerating system (10 mM isocitrate, 10 mM MgCl₂ and 0.08 U isocitrate dehydrogenase) in the assay mixture since large amounts of NADPH were consumed during the
assay (data not shown). The formation of omega-hydroxyphytanic acid followed simple Michaelis Menten kinetics (Fig. 3C). The apparent $K_m$ for NADPH derived from the Lineweaver-Burke plot (insert in Fig. 3C) was 35 $\mu$M.

Subsequently, we determined the effect of increasing phytanic acid concentrations on the formation of omega-hydroxyphytanic acid. To this end, different concentrations of phytanic acid were added in a fixed molar ratio between phytanic acid and methyl-beta-cyclodextrin (Fig. 3D). An apparent $K_m$ of $114 \pm 9 \mu$M was found.

Based on the experiments described above, we selected the following assay conditions: 0.75 mg/ml methyl-beta-cyclodextrin, 100 mM potassium phosphate (pH 7.6), 1 mM NADPH, and 200 $\mu$M phytanic acid. Under these conditions formation of omega-hydroxyphytanic acid was linear with time up to 60 min, and with protein up to 1 mg/ml (data not shown).

**Effect of imidazole derivatives on the formation of omega-hydroxyphytanic acid**

Imidazole antimycotics are known inhibitors of cytochrome P450 enzymes (Halpert, 1995; Zhang, 2002; Maurice, 1992). To measure the influence of four different imidazole derivatives on the formation of omega-hydroxyphytanic acid, we studied the effect of different concentrations of these compounds on the formation of omega-hydroxyphytanic acid (Fig. 4A) and (omega-1)-hydroxyphytanic acid (Fig. 4B). Fig. 4A shows that omega-hydroxyphytanic acid formation was inhibited by all four compounds with bifonazole as the most potent inhibitor, followed by ketoconazole, miconazole, and clotrimazole. Interestingly, a different picture was observed if the effect of the four imidazole derivatives was studied on the formation of the (omega-1)-compound with miconazole as most potent inhibitor, followed by ketoconazole and bifonazole. Remarkably, clotrimazole showed a stimulatory effect at low
concentrations with little inhibition at the highest concentrations used (100 µM).

To summarize, phytanic acid is hydroxylated to its omega and omega-1 hydroxy analogues in rat liver microsomes. The enzyme(s) responsible for phytanic acid omega- and (omega-1)-hydroxylation were shown to be NADPH dependent. Moreover, the formation of the omega- and omega-1 hydroxy analogues of phytanic acid was inhibited by imidazole antimycotics. The inhibition by the imidazole derivatives showed a different pattern for the two products. Hence, this strongly suggests that different members of the cytochrome P450 multi-enzyme family are responsible for the formation of omega- and (omega-1)-phytanic acid.
Example 2

In Example 1 we have shown that rat liver microsomes are able to omega-hydroxylate phytic acid. This reaction is catalyzed by at least one member of the cytochrome P450 enzyme family and results in the formation of two metabolites, omega- and (omega-1)-hydroxyphytanic acid. Cytochrome P450 enzymes are readily inducible by a variety of drugs (Waxman, 1999; Honkakoski, 2000). According to the present invention, induction of at least one cytochrome P450 involved in phytic acid omega-hydroxylation leads to an increased clearance of phytic acid in Refsum patients with obvious implications for the treatment of these patients. In this Example, we have extended our studies from rat liver microsomes to human liver microsomes.

Materials and Methods

Materials

Phytanic acid was obtained from the VU University Medical Center Metabolic Laboratory (Dr H.J ten Brink, Amsterdam, the Netherlands). 3-Hydroxyheptadecanoic acid was from Larodan Fine Chemicals AB (Malmö, Sweden). NADPH and NAD+ were obtained from Roche (Mannheim, Germany). Clotrimazole, ketoconazole, bifonazole and miconazole were obtained from Sigma (St. Louis, MO, USA). Methyl-beta-cyclodextrine was from Fluka (Buchs, Switzerland). N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). Pooled human liver microsomes were obtained from BD Gentest™ (Woburn, MA, USA). Rat liver microsomes were prepared from
Male Wistar rats by differential centrifugation as described in detail in Example 1. Other chemicals used were of the highest quality possible.

**Phytanic acid omega- and omega-1-hydroxylation assay**

Essentially the same conditions were used as described in Example 1, except for the final reaction volume which was now 0.1 ml. In brief, phytanic acid dissolved in DMSO was added to a solution of microsomes (1 mg/ml final concentration) in 100 mM potassium phosphate buffer (pH 7.7), containing phytanic acid at a final concentration of 200 μM, unless indicated otherwise.

Reactions were initiated by adding NADPH (final concentration 1 mM) and terminated by addition of 0.1 ml 1 M HCl. Subsequently, 0.5 ml phosphate-buffered saline (PBS) was added followed by 50 μL 12.1 M HCl. After addition of the internal standard (IS, 2 nmol 3-hydroxyheptadecanoic acid in 20 μl ethanol) the samples were extracted twice with 6 ml ethylacetate-diethylether (1:1 v/v). The organic layers were collected and the solvents evaporated. The residue was dissolved in 4 ml ethylacetate and further dried with anhydrous MgSO₄ and again evaporated. To enable gas chromatography-mass spectrometry (GS/MS) analysis the extracted fatty acids were derivatized to their corresponding trimethylsilyl (TMS) compounds essentially using the procedure described by Chalmers and Lawson (Chalmers, 1982). TMS ester/ether formation was performed by incubating the samples with 40 μl BSTFA containing 1% TMCS and 10 μl pyridine at 80°C for 1 hour. After the incubation the solution was directly used for GC-MS analysis.

**GC/MS**

GC/MS was performed on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 mass-selective detector (Palo Alto, CA). Samples (1 μl) were injected in the splitless mode (Hewlett Packard 7683 injector) and analyzed on a CP-Sil 5 CB low bleed MS column (25 m x 0.30...
μm) (Chrompack, Middelburg, The Netherlands). The oven temperature program used is the same as described in Example 1. The single ion monitoring (SIM) mode was applied for the detection of the respective (M-15)+ ions (m/z 369 and 457; masses of the molecular ions minus one methyl group of the TMS derivatives of phytanic acid and omega(-1)-hydroxyphytanic acid respectively). Analyte quantification was done by integration of the peaks followed by dividing the analyte peak areas by the area of the internal standard (TMS derivative of 3-hydroxyheptadecanoic acid, monitored ion m/z 233).

Results
Phytanic acid omega-hydroxylation in pooled human liver microsomes

Our studies show that phytanic acid undergoes NADPH-dependent omega-and (omega-1)-hydroxylation in rat liver microsomes (see Example 1). To test whether this also occurs in human liver microsomes the same assay was performed under the optimum conditions described for rat liver microsomes (see Materials and Methods section of Example 1). Under these conditions, the human liver microsomes indeed showed the capacity to produce omega- and (omega -1)-hydroxyphytanic acid (Fig. 5). The identity of the omega- and (omega-1)-hydroxylated products was confirmed by their corresponding fragmentation patterns as was done earlier when using rat liver microsomes (see Example 1). The major difference between rat and human microsomal systems is the ratio between the two products formed, as can be seen in Fig. 5. In pooled human microsomes the ratio omega: (omega-1) was 15.4 ± 0.7, whereas in rat liver microsomes this ratio was found to be 2.2 ± 0.2 as measured in four separate experiments.
Optimization of the phytic acid omega (-1)-hydroxylase assay
The marked difference between the ratios of product formation between
human and rat liver microsomes (Fig. 5) led us to optimize the hydroxylation
assay in human liver microsomes for omega-hydroxyphytanic acid formation.
First, we tested the effect of methyl-beta-cyclodextrin, which was used in the
assay to increase the solubilization of phytic acid. As shown in Fig. 6A,
methyl-beta-cyclodextrin had a positive influence on the formation of omega-
hydroxyphytanic acid up to approximately 1 mg/ml. A further increase of
methyl-beta-cyclodextrin in the assay had a negative effect on the rate of
product formation, presumably caused by a decrease in the availability of the
substrate. The same phosphate-based buffer system as used in Example 1 on
rat liver microsomes (50 mM potassium phosphate/ 50 mM pyrophosphate)
was used for the determination of the optimum pH of the reaction. The pH
optimum of the reaction was 7.7 (Fig. 6B). All subsequent experiments were
done at this particular pH.
In order to determine the Km for NADPH, the NADPH concentration was
varied in the assay in combination with the use of an NADPH-regenerating
system (10 mM isocitrate, 10 mM MgCl₂ and 0.08 U isocitrate
dehydrogenase). The reaction followed simple Michaelis Menten kinetics (Fig.
6C) and from the Lineweaver Burke plot (insert Fig. 6C) a Km of 2 µM (mean
of duplicate experiments) could be deduced. This Km is considerably lower
than the Km determined in rat liver microsomes (35 µM) (see Example 1).

Subsequently, the Km of the enzyme for phytic acid was determined.
Different concentrations of phytic acid were used with a fixed ratio
between phytic acid and methyl-beta-cyclodextrin. The v versus [S] plot did
not follow Michaelis-Menten kinetics so the calculation of the respective Km
from the Lineweaver Burke plot could not be done. Consequently, we
estimated the Km for phytic acid as the substrate concentration that shows
half maximal omega-hydroxylation activity. Based on the data in Fig. 6D, an apparent Km of 80 μM was determined (Fig. 6D). The nonlinear kinetics may, at least in part, be caused by inefficient solubilization of phytanic acid, although beta-methylcyclodextrin was used in the assay. *In vivo*, this problem is for instance overcome by a carrier protein which provides the substrate to the P450 enzyme, similar to sterol carrier protein 2 (SCP2) acting as a carrier protein for phytanoyl-CoA during alpha-oxidation (Mukherji, 2002). Liver fatty acid binding protein (L-FABP) is a possible candidate to play such a role during omega-hydroxylation based on the notion that L-FABP has a high affinity for phytanic acid outside of the peroxisome. L-FABP is already known to be required for regular branched-chain fatty acid uptake and metabolism by having a role in cytoplasmic fatty acid transport.

**Effect ofazole antimycotics**

The formation of omega-hydroxyphytanic acid was shown to be inhibited by imidazole derivatives in rat liver microsomes, indicating that the reaction is catalyzed by a member of the cytochrome P450 enzyme family. To establish whether omega-hydroxylation of phytanic acid is also catalyzed by a cytochrome P450 protein in human liver microsomes, we performed activity measurements in the presence of different concentrations of the imidazole derivatives bifonazole, clotrimazole, ketoconazole and miconazole, which were all found to inhibit product formation (Fig. 7A). Ketoconazole appeared to be the most potent inhibitor and not bifonazole which was most potent in rat liver microsomes (see Example 1). As shown in Fig. 7 the effect of the inhibitors on (omega-1)-hydroxyphytanic acid formation was much more pronounced as compared to the inhibitory effect on omega-hydroxyphytanic acid formation. This is also clear from the IC50 values in Table 1.
The results of Example 2 show that human liver microsomes are able to omega-hydroxylate phytanic acid. The formation of the products omega- and (omega-1)-hydroxyphytanic acid is NADPH dependent and inhibited by imidazole antifungal agents.

Example 3
Specific cytochrome P450 enzymes were tested for their capability of catalyzing omega-hydroxylation of phytanic acid with the following experiment:

Supersomes, in which each of the individual cytP450s were expressed were obtained from commercial sources (BD Gentest™ (Woburn, MA)). At the day of the experiment an aliquot was taken from each of the different supersome preparations, followed by incubation in a standard reaction medium, containing 100 mmol/L potassium phosphate pH 7.8, 1 mmol/L NADPH, 0.75 mg/milliliter methyl-β-cyclodextrine, and 0.2 mmol/L phytanic acid. Reactions were allowed to proceed for 30 minutes after which the formation of omega-hydroxy phytanic acid was quantified by means of gas chromatography and mass spectrometry analysis.

Example 4. Production of mutant mice

Construction of the targeting vector.
Genomic clones were isolated from a 129 SVJ mouse λ-FIX II genomic library (Stratagene) using complete mPhyH cDNA as a probe. Positive phages were further screened with various parts of mPhyH cDNA as probes and finally
two positive phages were selected, one containing the 5' end to exon 6-7 and one containing exon 6-7 to the 3' end of the PhyH gene. The two selected phages were purified and subsequently DNA was isolated, digested with NotI and subcloned into a low copynumber plasmid, pBR-GEM11, which was kindly provided by H. ten Riele (The Netherlands Cancer Institute, Amsterdam, The Netherlands). From both constructs, C4 and C11, a restriction map was made and, with various parts of mPhyH cDNA which were used as probes, the orientation of part of the gene in both constructs was determined. Based on the restriction maps, a large fragment of approximately 6.5 kb and a small fragment of approximately 2 kb were chosen to generate a targeting vector. A 4.4 kb BamHI-SmaI fragment containing exon 7-9 and 3' flanking sequence was subcloned from C11 and used to isolate a 1.8 kb HindIII/ClaI fragment ("short arm") that was ligated into a pBluescript-SK+ based plasmid which was already carrying the hygromycin B resistance gene. This plasmid then was linearized with XhoI, end-filled, cut with HindIII and ligated into pBluescript-SK+ (opened with HindIII and SmaI). The short arm and hygromycin B resistance gene were liberated from into pBluescript-SK+ by using HindIII and NotI and subsequently ligated into pBR-GEM11 opened with the same restriction enzymes. A 7.8 kb EcoRI fragment containing the 5' flanking sequence and exon 1-3 was subcloned from C4 and used to isolate a 6.7 kb EcoRI/XhoI fragment ("long arm") that was ligated into TOPO. The long arm was cut out TOPO by using NotI and XhoI and ligated into pBR-GEM11 containing the short arm and the resistance gene, which was opened with NotI and XhoI. For electroporation of ES cells either BamHI linearized targeting vector was used or the HindIII fragment containing the complete short arm, hygromycin B resistance gene and 6.3 kb of the long arm.
Culturing and electroporation of ES cells.
The IB10 subclone from the E14 ES cell line was obtained from The Netherlands Cancer Institute (Amsterdam, The Netherlands) and grown on irradiated murine embryonic fibroblast feeder cells in GMEM supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 x nonessential amino acids, 0.1 mM β-mercaptoethanol and 1000U/ml LIF in a 5% CO2-humidified incubator (REF Robanus). 6 x 10⁶ IB10 ES cells were electroporated with 30 μgram targeting DNA in a total volume of 300 μl PBS using a single pulse from a gene pulser (Bio-Rad) at 0.8 kV and 3 μF. Selection with hygromycin (150 μg/ml) was started the day after the electroporation. During selection the ES cells were cultured in 60% BRL-conditioned medium without feeder cells.

Screening of ES cells.
Targeted ES cells were screened by PCR for homologous recombination, using the primers 5'-CGCGAAGGGCCACAAAGAAC and 5'-TCCCCAGAAAGCAAGCAAAAGACT and an annealing temperature of 58 °C. These primers correspond to hygromycin B resistance marker and the 3' genomic flank outside of the construct, respectively. A fragment of the expected size of 2.0 kb was amplified in 12 out of 740 clones. PCR positive clones were also analyzed by southern blot for correct 5' homologous recombination. Genomic DNA was digested overnight with EcoRV and BamHI and hybridisation of the southern blot was carried out with an external 5' probe (EcoRI – HincII fragment, containing exon 1). This 5' external probe hybridizes to a 12.3 kb fragment from wild type. When one allele of the PhyH gene is replaced with the targeting vector sequences by homologous recombination, an additional fragment of 10 kb appears. Several ES clones which had undergone the correct homologous recombination were
verified for the correct karyotype (>12/15 metaphase chromosome spreads with 40 chromosomes).

5 **Generation and breeding of mutant mice.**
ES cells from three targeted clones were injected in C57BL/6 blastocysts, resulting in twelve chimeric mice obtained from two ES clones. Five chimeras, three derived from one ES clone two from another, showed germ-line transmission and were bred to FVB females. The grey offspring was analyzed by PCR on genomic ear DNA. The forward primer for the wild-type allele was 5'-CCTCTCCAATCTTAGTCGGTCCTTTCT. The forward primer for the targeted *PhyH* allele was located in the promoter in front of hygromycin: 5'-CCTACCGTGGATGTGGAATGTG. One reverse primer was used with both forward primers: 5'-AGCCCCCTAGCGTTTCCTCTGTGA at an annealing temperature of 59 °C. The PCR product obtained from the wild-type allele was 205 bp, from the targeted allele 262 bp. Mice carrying the mutant *PhyH* allele were crossed. All animal experiments were conducted under the approval of the animal care committee of the KNAW (Royal Dutch Academy of Arts and Sciences).

Animal feeding studies.
Six male mice, 8–9 weeks old, 25-30 gram were used for the phytol diet study. They were divided into 2 groups. One group was fed with 0.25% (w/w) phytol rodent chow diet (Arie Blok Diervoeding, Woerden, The Netherlands) the other group was fed with the identical rodent chow diet but without phytol. Equal number of male wild-type and heterozygous littermates were used for the study. All animals received water and food *ad libitum* for the treatment period of two weeks. They were kept individually, and food intake and body weight were monitored daily. At the end of the treatment, between 9 and 10
a.m., mice were sacrificed with CO₂ and tissues were dissected and immediately frozen in liquid nitrogen. For the isolation of blood samples to measure various parameters an identical animal study was carried out with the exception that the mice had been fasting 4 hours before they were sacrificed.

**Example 5**

The mice described above are fed with 0.25% (w/w) phytol rodent chow diet (Arie Blok Diervoeding, Woerden, The Netherlands). The mice are divided into two groups. One group additionally receives a candidate compound. The other group serves as a negative control. All animals receive water and food ad libitum for the treatment period. They are kept individually and food intake and body weight are monitored daily. At the end of the treatment the mice are sacrificed with CO₂ and tissues are dissected and immediately frozen in liquid nitrogen. It is subsequently determined whether less phytanic acid accumulation occurs in the mice which were provided with the candidate compound, as compared to the mice which were not provided with the candidate compound.

For the isolation of blood samples to measure various parameters an identical animal study is carried out with the exception that the mice have been fasting 4 hours before they are sacrificed. Phytanic acid will be measured in plasma and tissues of mice, which were provided with the candidate compound or not, by means of gas chromatography / mass spectrometry, and/or tandem mass spectrometry.

Based on the finding that the expression of CYP4A11 is induced by PPARalpha, which can be activated by a variety of specific ligands, including fibrates, such as fenofibrate, we have performed experiments in control and
phytanoyl-CoA hydroxylase deficient mice (model for Refsum disease) fed a diet containing 0.1% (W/W) fenofibrate. After the mice was sacrificed, the livers were removed, followed by preparation of post nuclear supernatants and measurement of the phytanic acid omega-oxidation capacity. These experiments revealed a 10-fold induction of the capacity to omega-oxidize phytanic acid.

Example 6

Introduction

The degradation of the majority of fatty acids occurs via β-oxidation in mitochondria and peroxisomes. 3-Methyl-branched-chain fatty acids, however, are not substrates for regular β-oxidation but first need to undergo one round of α-oxidation in peroxisomes. During α-oxidation a one-carbon unit is removed from the carboxy-terminus of the fatty acid yielding the n-1 analogue with the methyl group at position 2. The 2-methyl-branched-chain fatty acid thus formed, can then undergo normal β-oxidation.

The most abundant 3-methyl-branched chain fatty acid, at least in humans, is phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). Phytol, a fatty alcohol and a metabolite of chlorophyll, is the precursor of phytanic acid. Mammals cannot absorb chlorophyll and release phytol, but are able to convert free phytol into phytanic acid and to absorb phytanic acid itself. Fats of dairy products and meat are rich in phytanic acid and phytol since chlorophyll degradation and the subsequent conversion of phytol to phytanic acid occurs effectively by bacteria present in the rumen of ruminants. Phytanic acid accumulates in patients with adult Refsum Disease (ARD, MIM 266500) which is often due to a defect in the α-oxidation pathway
caused by mutations in one of two genes including the \textit{PAHX} gene which codes for phytanoyl-CoA hydroxylase, and/or the \textit{PEX7} gene which codes for the PTS2 receptor. The majority of ARD patients have mutations in the \textit{PAHX} gene. The increased levels of phytanic acid in plasma and tissues are thought to be the direct cause for the pathology of the disease. Among the symptoms are: progressive retinitis pigmentosa, peripheral neuropathy, anosmia, cerebellar ataxia and ichthyosis. The accumulation of phytanic acid is gradual because phytanic acid originates from dietary sources. The only known treatment of ARD consists of a diet low in phytanic acid, which may be combined with plasmapheresis.

Omega-oxidation (\(\omega\)-oxidation) leads to the formation of a carboxyl group at the \(\omega\)-end of the fatty acid. The first step involves hydroxylation of the fatty acid at the \(\omega\)-end of the molecule. The \(\omega\)-hydroxylated fatty acid is then converted into the corresponding dicarboxylic acid, which may either be catalysed by the subsequent action of an alcohol dehydrogenase and an aldehyde dehydrogenase, or catalysed by a cytochrome P450 (CYP450) enzyme. In the case of phytanic acid, \(\omega\)-oxidation introduces a new carboxyl group on the \(\omega\)-end with a methyl-group at position 2. The formed \(\omega\)-dicarboxylic acid of phytanic acid, i.e. phytanedioic acid, undergoes \(\beta\)-oxidation after activation of the \(\omega\)-carboxygroup to its CoA-ester in the same manner as 2-methyl-branched fatty acids.

We have shown that the first step of the \(\omega\)-oxidation pathway, i.e. the \(\omega\)-hydroxylation of phytanic acid, takes place in rat and human liver microsomes and is catalyzed by one or more members of the CYP450 enzyme superfamily. The expression of many of the enzymes which belong to this family is known to be induced by a large variety of drugs. Hence, induction of the CYP450 responsible for the \(\omega\)-hydroxylation of phytanic acid increases the flux through the \(\omega\)-oxidation pathway, thereby increasing the clearance of phytanic acid in ARD patients. As the CYP450 enzymes have multiple roles
in cellular metabolism, the induction of the CYP450 involved is preferably as specific as possible in order to better avoid or reduce possible unwanted side effects from the administered drugs. For this reason it is desired to identify the specific CYP450 involved in the ω-hydroxylation of phytanic acid. In this example we report our studies on the identification of the CYP450 enzyme(s) involved in the ω-hydroxylation of phytanic acid in humans.

**Materials and methods**

Phytanic acid was obtained from the VU University Medical Center, Metabolic Laboratory (Dr H.J ten Brink, Amsterdam, the Netherlands). 3-Hydroxyheptadecanoic acid was from Larodan Fine Chemicals AB (Malmö, Sweden). NADPH and NAD⁺ were obtained from Roche (Mannheim, Germany). Methyl-β-cyclodextrine was from Fluka (Buchs, Switzerland). N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). Pooled human liver microsomes and human recombinant CYP450 isoforms (Supersomes™) were obtained from BD Gentest™ (Woburn, MA, USA). The CYP450 inhibitors diethylidithiocarbamate (DDC), furafylline, ketoconazole, 17-octadecynoic acid (17-ODYA), omeprazole, quinidine, sulphaphenazole, trimethoprim, and troleandomycin were purchased from Sigma Aldrich (St Louis, MO, USA). All other chemicals used were of the highest quality possible.

The ω-hydroxylation of phytanic acid was measured as described in Examples 1 and 2 with slight modifications. In brief, phytanic acid dissolved in DMSO was added to a reaction mixture (final volume 0.2 ml) containing human liver microsomes (1 mg/ml final concentration), and 100 mM potassium phosphate buffer (pH 7.7) containing methyl-β-cyclodextrin (0.75 mg/ml final concentration). When Supersomes™ (25 pmol P450/ml sample) were used in
experiments, the protein concentration of the reaction mixture was kept constant at 0.75 mg/ml by adding Insect Control Supersomes™. The final concentration of phytanic acid was 0.2 mM unless indicated otherwise. Reactions were initiated by the addition of NADPH (final concentration 1 mM) and terminated after 30 min by adding 0.2 ml of 1 M HCl to the mixture. Subsequently, 1 ml phosphate-buffered saline was added, followed by 100 μL 12.1 M HCl, and 20 μl 0.2 mM 3-hydroxyheptadecanoic acid in DMSO as internal standard. The reaction mixtures were extracted twice with 4 ml ethylacetate-diethylether (1:1 v/v). Both fractions were collected and the solvents were evaporated under N₂. The extracted fatty acids were derivatized to their corresponding trimethylsilyl derivatives for gas chromatography/mass spectrometry analysis as described in Example 1. During the inhibitor studies the ω-hydroxylation assay was performed as described above except that incubations were performed in the presence of the following inhibitors: trimethoprim (CYP2C8), sulfaphenazole (CYP2C9), omeprazole (CYP2C19), quinidine (CYP2D6), DDC (CYP2E1), and ketoconazole (CYP3A4) (Eagling et al, 1998; Wen et al, 2002; Newton et al, 1995; Ko et al, 1997). Furafylline (CYP1A2), DDC (diethylthiocarbamate) (CYP2E1), 17-ODYA (17-Octadecynoic acid) (CYP4), and troleandomycin (CYP3A4) were pre-incubated with human liver microsomes for 15 minutes in the presence of NADPH before adding phytanic acid to the mixture and subsequent incubation (30 min). Stock solutions were prepared in DMSO. Inhibitor concentrations are given in Fig. 8.

**Results**

Recently, we showed that phytanic acid is ω-hydroxylated in (pooled) human liver microsomes and that this reaction is catalyzed by one or more CYP450 enzymes (Example 2). Furthermore, we found that phytanic acid is not exclusively hydroxylated at the ω-position but also at the (ω-1)-position in
a 15:1 ratio. In the present study we have focused only on the formation of ω-hydroxyphytanic acid because this compound is formed predominantly and can be converted into 1,16-phytanedioic acid, a substrate for subsequent β-oxidation.

To determine which CYP450 contributes to phytanic acid ω-hydroxylation in human liver microsomes, we have incubated human liver microsomes with a number of isoform-selective inhibitors as described in the materials and methods section (Eagling et al., 1998; Wen et al., 2002; Newton et al., 1995; Ko et al., 1997). Substantial inhibition of phytanic acid ω-hydroxylation was only found with ketoconazole, DDC, and 17-ODYA, which are specific inhibitors of CYP3A4, CYP2E1 and CYP4 family members, respectively. Troleandomycin, omeprazole, and trimethoprim showed minor effects, while with sulphaphenazole, quinidine, and furafylline no inhibitory effect was observed at all (Fig. 8). Inspection of the results of Fig. 8 reveals that ketoconazole is a relatively poor inhibitor of phytanic acid ω-hydroxylation (IC50 value > 1 μM). Indeed, the IC50 value of ketoconazole as determined for the CYP3A4 enzyme is in the nanomolar range. The inhibitory effect of ketoconazole at higher concentrations is probably due to the fact that at these concentrations ketoconazole also inhibits other CYP450s.

In order to estimate the IC50 values for the inhibitors DDC and 17-ODYA, we incubated human liver microsomes in the presence of phytanic acid and different concentrations of inhibitor (Fig. 9). From the data of Fig. 9A, an IC50 value of DDC of 90 μM can be calculated (Fig. 9A). Although DDC is often used as a selective inhibitor of CYP2E1 in literature, DDC is actually a more potent inhibitor of CYP2A6 and CYP2B6 catalyzed reactions (IC50 ≈ 125 μM for CYP2E1 vs. IC50 ≤ 10 μM for CYP2A6 and CYP2B6). It is concluded from these IC50 values that the contribution of CYP2A6 and 2B6 to the ω-hydroxylation of phytanic acid is only minor, whereas CYP2E1 cannot be excluded from having phytanic acid ω-hydroxylation activity.
However, since DDC is known to inhibit multiple CYP450 isoforms when used in high concentrations it may well be that multiple CYP450s are involved in the inhibitory action of DDC in addition to CYP2E1. As shown in Fig. 9B, 17-ODYA was found to be a potent inhibitor of phytanic acid ω-hydroxylation with an IC50 < 400 nM. 17-ODYA is a fatty acid analogue which acts through suicide inhibition and is selective for the CYP4 family of ω-hydroxylases (IC 50 < 100 nM [23]) which are involved in the ω-hydroxylation of long-chain fatty acids, arachidonic acid and leukotrienes.

The results described in the previous paragraph show that one or more members of the CYP4 family are involved in the ω-hydroxylation of phytanic acid because the IC50 value found for 17-ODYA is comparable with previously reported values for this family of CYP450 enzymes. In order to substantiate this conclusion, further confirmation for this, phytanic acid was incubated with microsomes containing individually expressed CYP4s prepared from baculovirus-infected insect cells. To this end, we tested all commercially available members of the human CYP4 family. Furthermore, we also performed incubations with Supersomes™ containing CYP3A4 and CYP2E1 to verify the conclusion that the inhibition observed with ketoconazole and DDC was not specific (see previous paragraph). Indeed, as shown in Fig. 10, microsomes expressing CYP3A4 and CYP2E1 were completely devoid of phytanic acid ω-hydroxylation activity. Among the CYP4s tested, there were four CYP450s displaying phytanic acid ω-hydroxylation activity: CYP4F3A, CYP4F3B, CYP4A11 and CYP4F2 (Fig. 10). In our effort to determine the kinetic parameters of the different CYP4s we varied the substrate concentration in the incubation mixture with the four active CYP4s. However, the data points of Fig. 11 did not allow determination of the Km constants due to the non-linearity of the corresponding Lineweaver-Burke plots (not shown). From Fig. 11, the Km values can be estimated as half of the Vmax values. The Km values deduced in this way for the four active CYP4s are about the same (± 75 μM). Hence,
CYP4F3A is the most efficient CYP4 in \( \omega \)-hydroxylation of phytanic acid because it has the highest apparent \( V_{\text{MAX}} \) value while the \( K_{\text{M}} \) values for all active CYP4 enzymes are approximately equal and therefore CYP4F3A has the highest \( V_{\text{MAX}}/K_{\text{M}} \) ratio.

Discussion

The results described in this Example show that among the CYP4 family members tested in this study, CYP4F3A is the most efficient enzyme in \( \omega \)-hydroxylation of phytanic acid followed by CYP4F3B. CYP4F3B is a splice variant of the \textit{CYP4F3} gene in liver and differs in only one exon from CYP4F3A (Christmas et al, 1999). This has been shown to cause a difference in substrate specificity with CYP4F3A having the highest affinity for leukotriene B4 (LTB4) and CYP4F3B for arachidonic acid (Christmas et al, 2001). In this Example the difference in substrate specificity between the two splice variants is shown by using phytanic acid as a substrate.

The CYP4F3A enzyme is expressed in polymorphonuclear leukocytes and was found to be involved in LTB4 \( \omega \)-hydroxylation, a process required for the degradation of this inflammatory agent. Since CYP4F3A is not expressed in liver, this enzyme cannot be responsible for the phytanic acid \( \omega \)-hydroxylation activity observed in human liver microsomes. Based on the results described in this Example other liver CYP4 enzymes able to \( \omega \)-hydroxylate phytanic acid in addition to CYP4F3B are CYP4A11 and CYP4F2. The CYP4A family is known to be induced by hypolipidemic drugs and peroxisome proliferators (PP) via PPAR\( \alpha \), and is therefore considered as a potential target for inducing the activity of the \( \alpha \)-oxidation pathway of phytanic acid in ARD patients.
Example 7

In this Example, we investigate the very long-chain fatty acids (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-linked adrenoleukodystrophy (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), CYP4F12 (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 Example to study the hydroxylation of different VLCFAs were adapted from previous experiments with minor modifications (Sanders et al, 2005). 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 (Valianpour et al, 2003; Sanders et al, 2005).

**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 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,

\[
V = \frac{V_{\text{max1}} \cdot S}{K_{m1} + S} + \frac{V_{\text{max2}} \cdot S}{K_{m2} + S}
\]

where $K_{m1}$ and $K_{m2}$ are the high and low affinity Michaelis-Menten constants, respectively, and $V_{\text{max1}}$ and $V_{\text{max2}}$ 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 (Korzekwa et al, 1998):
\[
V = \left( \frac{V_{\text{max1}} \cdot S + V_{\text{max2}} \cdot S^2}{K_{m1} \cdot K_{m2}} \right) \left( 1 + \frac{S}{K_{m1}} + \frac{S^2}{K_{m1} \cdot K_{m2}} \right)
\]

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

10  Optimization of the VLCFA hydroxylation assay

Previous studies have demonstrated that C22:0 is a substrate for the \(\omega\)-oxidation system in rat liver microsomes (sanders et al, 2005). To study the \(\omega\)-hydroxylation capacity of human liver microsomes for VLCFAs, the hydroxylation assay was optimized for the \(\omega\)-oxidation of C26:0. To this end, human microsomal protein was incubated in a buffered medium containing NADPH and \(\alpha\)-cyclodextrin to solubilize C26:0. The production of \(\omega\)-hydroxy-C26:0 was linear with time up to 30 min (Figure 12A). 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 12B shows that the formation of \(\omega\)-hydroxy-C26:0 was maximal at pH 8.4.

Interestingly, the oxidation of C26:0 was not limited to the production of \(\omega\)-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 12A, B). Total product formation was linear with protein up to 60 µg/ml (Figure 12C). 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 Heps. Hydroxylation of C26:0 was maximal with Tris as buffer (results not shown), which has therefore been used in subsequent experiments.

10 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 13). 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 13. The kinetic parameters calculated from the experimental data of each of the different substrates using this model are listed in Table 3. 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 3 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 \( \omega \)-oxidation products

Hydroxylation of VLCFAs by cytochrome P450 enzymes may occur at the \( \omega \)-position as well as at the adjacent positions. 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. 14). The major peak (Fig. 14, peak II) with a retention time of 14.5 min was analyzed by mass spectral analysis and identified as \( \omega \)-hydroxy-C26:0 (Fig 15A). The fragment at m/z 103 is characteristic of \( \omega \)-hydroxy fatty acids, representing the terminal CH\(_2\)OSi(CH\(_3\))\(_3\) moiety (Jakobs et al, 1981). 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. 14 peak I) was identified by mass spectral analysis as (\( \omega \)-1)-hydroxy-C26:0 (Fig. 15B). It was deduced from the mass spectrum that the peak at m/z 117 represents the CH\(_3\)CHO\(\)Si(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 \( \omega \) and (\( \omega \)-1) were not detected. In addition, the third peak (Fig. 14, peak III) with retention time of 14.85 min was identified as C26:0-DCA (Fig. 15C). 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 \( \omega \)/ (\( \omega \)-1)-hydroxylation for each substrate is listed in Table 3. 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. 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), quinidine (for CYP2D6), ketoconazole (for CYP3A), troleandomycin (for CYP3A4), furafylline (for CYP1A2), DDC (for CYP2E1), trimethoprim (for CYP2C8) and 17-ODYA (for CYP4A/F). In Figure 16, 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 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 show that cytochrome P450 enzymes belonging to the CYP2E1, CYP3A and CYP4A/F subfamily are 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 reveal that CYP4F2 as well as CYP4F3B catalyze the hydroxylation of C26:0 (Figure 17). 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 18 and 19 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. 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 18 and 19. The kinetic parameters calculated for each of the different substrates are listed in Table 4. 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 extent the (ω-1)-group (data not shown). The ω: (ω-1)-hydroxylation ratio for each substrate is listed in Table 4.
Discussion

In this Example, we investigated whether the ω-oxidation pathway 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. Based on the inhibition studies (Fig. 16) and the experiments with Supersomes™ containing individual human cytochrome P450 enzymes (Fig. 17), 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. 18/19). 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.

Example 8

As an alternative to the pharmacological induction of the CYP450 in question, increased levels of the CYP450 in question are achieved via gene therapy. Preferably, cDNA encoding the CYP450 in question is integrated into a vector specific for the liver such as an AAV-vector using methods well known in the art, in order to achieve direct targeting of the CYP450 in question to the liver.
Brief description of the drawings

Figure 1. GC-MS (single-ion monitoring mode) chromatograms of extracts from rat liver microsomes incubated with phytanic acid in the absence (A) or presence (B) of NADPH. Spectrum analysis was done as described in Materials and Methods of Example 1. Peaks corresponding to the \((M-15)^+ (m/z = 457)\) of hydroxylated phytanic acid metabolites are labelled I and II. 3-Hydroxyheptadecanoic acid (ion \(m/z = 233\)) was used as internal standard (IS).

Figure 2. Mass spectra of peaks labelled I and II in Fig. 1B. Based on the fragmentation pattern shown in (A) and (B), peak I was identified as the trimethylsilyl (TMS) derivative of (omega-1)-hydroxyphytanic acid, whereas peak II was identified as the TMS-derivative of omega-hydroxyphytanic acid.

Figure 3. Optimization of the phytanic acid hydroxylase activity in rat liver microsomes.
A: The effect of different concentrations of methyl-beta-cyclodextrin on the formation of omega-hydroxyphytanic acid (omega-HPA) in rat liver microsomes was determined under conditions described in Materials and Methods of Example 1.

B: The pH dependency of phytanic acid omega-hydroxylation. The hydroxylase assay was performed as in A, with the exception of the use of a combined buffer containing 50 mM potassium phosphate and 50 mM pyrophosphate and 0.75 mg/ml methyl-beta-cyclodextrin.

C: The effect of the NADPH concentration on phytanic acid omega-hydroxylation in the presence of a NADPH regenerating system (10 mM isocitrate, 10 mM MgCl\(_2\) and 0.08 U isocitrate dehydrogenase). The experimental set-up was as described in Materials and Methods of Example
1, with the exception of using a 100 mM potassium phosphate buffer (pH 7.6) and 0.75 mg/ml methyl-beta-cyclodextrin. The $K_m$ for NADPH was determined to be 35 $\mu$M as derived from the Lineweaver Burke plot (insert). The effect of the phytanic acid concentration on the formation of omega-hydroxyphytanic acid was determined using the optimum experimental conditions derived from the previous experiments [100 mM potassium phosphate buffer (pH 7.6), 1 mM NADPH]. The ratio methyl-beta-cyclodextrin to phytanic acid was kept constant. All data shown represent means of duplicate experiments with the exception of Figure 3C, in which 3-4 separate experiments were done with the SD values shown as error bars.

Figure 4. Effect of different imidazole antifungal agents on the omega- and (omega-1)-hydroxylation of phytanic acid. Rat liver microsomes were incubated with phytanic acid in the presence of different concentrations of imidazole derivatives. The inhibitory effect of the imidazole derivatives on the formation of omega-hydroxyphytanic acid (omega-HPA; A) and (omega-1)-hydroxyphytanic acid (B) is shown. The data shown represent means of duplicate experiments.

Figure 5. GC-MS (SIM mode) chromatograms of extracts of human liver microsomes incubated with phytanic acid in the absence (x) or presence (squares) of NADPH. For comparison, a chromatogram of an extract of rat liver microsomes incubated with phytanic acid in the presence of NADPH (circles) is shown. The peaks are the (M-15)$^+$ ions ($m/z = 457$) of trimethylsilylated omega- and (omega-1)-hydroxyphytanic acid (omega- and (omega-1)-HPA).

Figure 6. Optimization of phytanic acid omega-hydroxylation in pooled human liver microsomes. (A) The effect of different concentrations of methyl-beta-cyclodextrin on the formation of omega-hydroxyphytanic acid
(omega-HPA) in rat liver microsomes was determined. The pH dependency of phytanic acid omega-hydroxylation is shown in (B). The hydroxylase assay was essentially performed as in (A), with the exception of the use of a combined buffer containing 50 mM potassium phosphate and 50 mM pyrophosphate and 0.75 mg/ml methyl-beta-cyclodextrin. The effect of the NADPH concentration on phytanic acid omega-hydroxylation in the presence of an NADPH-regenerating system (10 mM isocitrate, 10 mM MgCl₂ and 0.08 U isocitrate dehydrogenase) is shown in (C). The experimental set-up was as described in the Materials and Methods section of Example 2 with the exception of the use of a 100 mM potassium phosphate buffer (pH 7.7) and 0.75 mg/ml methyl-beta-cyclodextrin. The Kₘ for NADPH was 2 μM as determined from the Lineweaver Burke plot (insert). The effect of the phytanic acid concentration on the formation of omega-hydroxyphytanic acid (D) was determined using the optimum experimental conditions derived from the previous experiments (100 mM potassium phosphate buffer (pH 7.7), 1 mM NADPH). The ratio methyl-beta-cyclodextrin / phytanic acid was kept constant. The data shown represent means of duplicate experiments.

Figure 7. Effect of different imidazole antimycotics on the omega- and (omega-1)-hydroxylation of phytanic acid. Pooled human liver microsomes were incubated with phytanic acid in the presence of different concentrations of imidazole derivatives. The inhibitory effect of the imidazole derivatives on the formation of omega-hydroxyphytanic acid (A) and (omega-1)-hydroxyphytanic acid (B) is shown. The data shown represent means of duplicate experiments.

Figure 8. Inhibition of ω-hydroxyphytanic acid formation in human liver microsomes by several CYP450 isoform specific inhibitors. Pooled human liver microsomes were incubated with phytanic acid in the presence of the following inhibitors: TMP, trimethoprim (100 μM); Ket, ketoconazole (1 μM);
OP, omeprazole (10 µM); Sulf, sulfaphenozole (10 µM); Q, quinidine (10 µM); F, furafylline (20 µM); TOA, troleandomycin (100 mM); DDC, diethylctyliothiocarbamate (100 µM); 17ODYA, 17-octadecynoic acid (1 µM). The bars represent the mean values of duplicate experiments.

Figure 9. Inhibition of the formation of ω-hydroxyphytanic acid by different concentrations of DDC (A) and 17-ODYA (B). The inhibitory effect of DDC (A) and 17-ODYA (B) on the formation of ω-hydroxyphytanic acid in pooled human liver microsomes is shown. The data represents the mean of duplicate experiments.

Figure 10. ω-Hydroxyphytanic acid formation by human recombinant CYP450 isoforms. Microsomes containing individually expressed human CYP450 isoforms prepared from baculovirus-infected insect cells (Supersomes™) were incubated with phytanic acid in order to determine their phytanic acid ω-hydroxylation capacity. The bars represent the mean values of duplicate experiments.

Figure 11. ω-Hydroxyphytanic acid formation as a function of the amount of substrate in the assay. Supersomes™ were incubated in the presence of different concentrations of phytanic acid and NADPH under conditions described in the Methods section. The ratio methyl-β-cyclodextrin / phytanic acid was kept constant. The data points represent means of triplicates ± SD. (CYP4F3A (■), CYP4F3B (○), CYP4F2 (○), CYP4A11 (▲))

Figure 12. 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' of Example 7. Symbols used: (■) Total product formation, (●) ω-hydroxy-C26:0 and (●) C26:0-DCA.

Figure 13. 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' of Example 7. 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.

Figure 14. 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 of Example 7. 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).

Figure 15. Characterization of the C26:0 ω-oxidation intermediates from the peaks in Fig. 14. 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.

Figure 16. 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, diethylthiocarbamate; TMP, trimethoprim; 17-ODYA, 17-octadecynoic acid.

Figure 17. 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.

Figure 18. 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 37°C. The results are the mean of two independent experiments. Symbols used: (●) ω-hydroxy fatty acid and (♦) dicarboxylic acid.

Figure 19. 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.
**Table 1**

**Ligands of nuclear receptor family members**

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<thead>
<tr>
<th>Nuclear receptor ligands</th>
<th>Nuclear receptors affected by ligands</th>
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<tr>
<td>Oestrogens, retinoids</td>
<td>ERs, RARs, RXRs</td>
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<tr>
<td>Androgens</td>
<td>AR</td>
</tr>
<tr>
<td>Fatty acid derivatives</td>
<td>PPARs</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>AR, ER</td>
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<td>AR, ER, GR, MR</td>
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<td>Retinoic acid</td>
<td>RARs, RXRs</td>
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<td>Oxysterols, sterols</td>
<td>LXR, FXR</td>
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**Table 2**

IC50 values for the inhibition of phytanic acid omega- and (omega-1)-hydroxylation by imidazole derivatives as calculated from Figure 7

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 value omega-hydroxyphytanic acid</th>
<th>IC50 value (omega-1)-hydroxyphytanic acid</th>
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<tr>
<td>Bifonazole</td>
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<td>Clotrimazole</td>
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<tr>
<td>Miconazole</td>
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<td>&lt;2 μM</td>
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* Data represent the mean of duplicate experiments
Table 3
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.

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<th>Low affinity</th>
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<th></th>
<th></th>
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<td>$V_{max2}$ nmol min⁻¹ mg⁻¹</td>
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<td>Table 4</td>
<td>Kinetic parameters for VILCA hydroxylation by CYP4F2 and CYP4F3B. 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.</td>
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References


Claims

1. A method for at least in part counteracting a disease involving accumulation of a fatty acid, the method comprising administering a compound which is capable of inducing and/or upregulating omega-oxidation of said fatty acid, or whose metabolite is capable of inducing and/or upregulating omega-oxidation of said fatty acid, to a subject suffering from, or at risk of suffering from, said disease.

2. Use of a compound capable of inducing and/or upregulating omega-oxidation of a fatty acid, or whose metabolite is capable of inducing and/or upregulating omega-oxidation of a fatty acid, for the manufacture of a medicament for at least in part treating a disease involving accumulation of said fatty acid.

3. A method or a use according to claim 1 or 2, wherein said disease comprises a peroxisomal and/or mitochondrial fatty acid oxidation deficiency.

4. A method or a use according to any one of claims 1-3, wherein said disease comprises diabetes mellitus, medium chain acyl-CoA dehydrogenase deficiency, Refsum disease and/or X-linked adrenoleukodystrophy.

5. A method or a use according to any one of claims 1-4, wherein said compound comprises a cytochrome P 450 enzyme or a functional part, derivative and/or analogue thereof, and/or a nucleic acid sequence encoding a cytochrome P 450 enzyme or a functional part, derivative and/or analogue thereof.

6. A method or a use according to any one of claims 1-5, wherein said compound capable of upregulating omega-oxidation comprises a compound capable of increasing the amount of a cytochrome P450 enzyme.

7. A method or a use according to any one of claims 1-6, wherein said compound capable of upregulating omega-oxidation comprises a compound capable of enhancing the omega-hydroxylation capacity of a cytochrome P450 enzyme.
8. A method or a use according to claim 6 or 7, wherein said cytochrome P450 enzyme comprises a human cytochrome P450 enzyme.

9. A method or a use according to claim 8, wherein said human cytochrome P450 enzyme comprises CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B.

10. A method or a use according to claim 8, wherein said human cytochrome P450 enzyme comprises CYP4F2 and/or CYP4F3B.

11. A method or a use according to any one of claims 1-10, wherein said compound is capable of inducing and/or enhancing a cytochrome P450-specific promoter.

12. A method or a use according to any one of claims 1-11, wherein said compound comprises a ligand of a member of the nuclear hormone family.

13. A method or a use according to claim 12, wherein said compound comprises a ligand of PPARα, LXR, FXR, PXR, and/or CAR.

14. A method or a use according to any one of claims 1-13, wherein said compound comprises a fibrate, rifampicin and/or phenytoin.

15. A method or a use according to any one of claims 1-14, wherein said compound comprises bezafibrate, fenofibrate, gemfibrozil, and/or ciprofibrate.

16. A method or a use according to any one of claims 1-15, wherein said compound comprises a compound as depicted in Table 1.

17. A method or a use according to any one of claims 1-16, wherein said fatty acid comprises phytanic acid and/or C26:0.

18. Use of a non-human animal suffering from, or at risk of suffering from, a disease involving accumulation of a fatty acid for determining whether a compound is capable of inducing and/or upregulating omega-oxidation of said fatty acid.

19. A method for determining whether and/or to what extent a compound is capable of inducing and/or upregulating omega-oxidation of a fatty acid, comprising:

- providing a non-human animal suffering from, or at risk of suffering from,
a disease involving accumulation of said fatty acid with said fatty acid and
with said compound,
- determining the extent of accumulation of said fatty acid in said animal, and
- comparing said extent of accumulation with the extent of accumulation of
said fatty acid in the same kind of non-human animal which is not, or to a
significantly lesser extent, provided with said compound.

20. A use or a method according to claim 18 or 19, wherein said fatty acid
comprises C26:O or phytic acid.

21. Use of a compound which is capable of increasing the amount and/or the
omega-hydroxylation capacity of CYP4A11, CYP4F2, CYP4F3A and/or
CYP4F3B, or whose metabolite is capable of increasing the amount and/or the
omega-hydroxylation capacity of CYP4A11, CYP4F2, CYP4F3A and/or
CYP4F3B, for the preparation of a medicament for at least in part treating
Refsum disease.

22. Use of a compound which is capable of increasing the amount and/or the
omega-hydroxylation capacity of CYP4F2 and/or CYP4F3B, or whose
metabolite is capable of increasing the amount and/or the omega-
hydroxylation capacity of CYP4F2 and/or CYP4F3B, for the preparation of
a medicament for at least in part treating X-linked adrenoleukodystrophy.

23. A method for at least in part treating Refsum disease the method
comprising administering a compound which is capable of increasing the
amount and/or the omega-hydroxylation capacity of CYP4A11, CYP4F2,
CYP4F3A and/or CYP4F3B, or whose metabolite is capable of increasing
the amount and/or the omega-hydroxylation capacity of CYP4A11, CYP4F2,
CYP4F3A and/or CYP4F3B, to a subject suffering from, or at risk of
suffering from, Refsum disease.

24. A method for at least in part treating X-linked adrenoleukodystrophy the
method comprising administering a compound which is capable of
increasing the amount and/or the omega-hydroxylation capacity of CYP4F2
and/or CYP4F3B, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F2 and/or CYP4F3B, to a subject suffering from, or at risk of suffering from, X-linked adrenoleukodystrophy.

25. A kit comprising a compound capable of increasing the amount and/or the omega-hydroxylation capacity of a first enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of a first enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, and a compound capable of increasing the amount and/or the omega-hydroxylation capacity of a second enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of a second enzyme selected from the group consisting of CYP4A11, CYP4F2, CYP4F3A and/or CYP4F3B.

26. A kit comprising a compound capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F2, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F2, and a compound capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F3B, or whose metabolite is capable of increasing the amount and/or the omega-hydroxylation capacity of CYP4F3B.
Figure 1

A

MS output

Retention time (min)

B

MS output

Retention time (min)

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Figure 3

A: 

ω-HPA formation (fold increase) with [Methyl-β-Cyclodextrin] in mg/ml.

B: 

ω-HPA formation (nmol/min/mg) vs pH.

C: 

ω-HPA formation (nmol/min/mg) with [NADPH] in μM.

D: 

ω-HPA formation (nmol/min/mg) with [phytanic acid] in μM.

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Figure 4

A

$\omega$-HPA formation (% activity)

[inhibitor] in $\mu$M

Ketoconazole
Bifonazole
Clotrimazole
Miconazole

B

$(\omega-1)$-HPA formation (% activity)

[inhibitor] in $\mu$M

Ketoconazole
Bifonazole
Clotrimazole
Miconazole

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Figure 6

A: [Graph showing the relationship between fold increase and methyl-β-cyclodextrin (mg/ml).]
B: [Graph showing the relationship between pH and HPA formation (pmol/mg/min).]
C: [Graph showing the relationship between 1/[NADPH] in µM and [NADPH] in µM.]
D: [Graph showing the relationship between HPA formation (pmol/mg/min) and phytic acid in µM.]
Figure 7

Graph 1: % ω-hydroxylation activity vs. [Inhibitor] in μM

Graph 2: % (ω-1)-hydroxylation activity vs. [Inhibitor] in μM

Key:
- Ketoconazole
- Bifonazole
- Clotrimazole
- Miconazole
Figure 8

17ODYA
- CYP4A11
- CYP4F3
- CYP4F2

DDC
- CYP2E1

Ket
- CYP2C18
- CYP219
- CYP3A4

TOA
- CYP3A4

OP
- CYP219

TMP
- CYP2C8

F
- CYP1A2

Q
- CYP2D6

Sulf
- CYP2C9

% inhibition
Figure 10

ω-Hydroxyphytanic acid formation (pmol/min/pmol CYP450)

- CYP4F3A
- CYP4F3B
- CYP4F2
- CYP4A11
- CYP4F12
- CYP2E1
- CYP2J2
- CYP3A4
Figure 11
Figure 12

A

B

C
Figure 13

A

Activity (nmol/min/mg)

C22:0 (μM)

B

Activity (nmol/min/mg)

C24:0 (μM)

C

Activity (nmol/min/mg)

C26:0 (μM)
Figure 16
Figure 17

Product formation (pmol)

- CYP 4F12
- CYP 4F3B
- CYP 4F3A
- CYP 4F2
- CYP 4A11
- CYP 3A4
- CYP 2J2
- CYP 2E1
- Control
Figure 18

A

B

C

activity (pmol min⁻¹ pmol PAP⁻¹)

activity (pmol min⁻¹ pmol PAP⁻¹)

activity (pmol min⁻¹ pmol PAP⁻¹)

C22:0 (µM)

C24:0 (µM)

C26:0 (µM)