Role of Bile Acid Pathway Intermediates in Pathology of CTX

William J. Griffiths, Eylan Yutuc, Mohsen Ali Asgari and Yuqin Wang

Swansea University Medical School, ILS1 Building, Singleton Park, Swansea SA2 8PP, Wales, UK

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Abstract

A deficiency in the enzyme sterol 27-hydroxylase (CYP27A1) leads to the autosomal recessive disorder cerebrotendinous xanthomatosis (CTX). CYP27A1 catalyses the first steps of the acidic pathway of bile acid biosynthesis. Most cells express CYP27A1, and a deficiency in this enzyme results in the activation of shunt pathways to help remove excess cholesterol. CYP27A1 also appears in the middle of the neutral pathway of bile acid biosynthesis and its deficiency results in accumulation of up-stream pathway intermediates. Here we describe methods for the simultaneous analysis of almost all metabolites from cholesterol to bile acids in a single assay and discuss the potential importance of accumulation of pathway intermediates and missing metabolites to the pathology of CTX.

Introduction

The identification of sterol 27-hydroxylase (cytochrome P450 family 27 subfamily A member 1, CYP27A1, MIM 606530) as the deficient enzyme in the autosomal recessive genetic disorder cerebrotendinous xanthomatosis (CTX) was made thanks to the pioneering work of Ingemar Björkhem and David Russell in the 1980's and 1990's (1-3). CYP27A1 represents the first enzyme of the acidic pathway of bile acid synthesis introducing a hydroxy group at the terminal carbon of the sterol side-chain with resultant R stereochemistry at C-25 and further converts this primary alcohol to a carboxylic acid (Figure 1) (4). The enzymatic products 27-hydroxycholesterol (27-HC), systematically but less commonly named (25R)26-hydroxycholesterol (5), and 3β-hydroxycholest-5-en-(25R)26-oic acid (3β-HCA) are then metabolised through multiple steps to primary bile acids, mostly chenodeoxycholic acid (CDCA) (4). (Note, stereochemistry at C-25 in C_{27} sterols is assumed to be 25R unless stated otherwise). CYP27A1 also appears in the neutral pathway of bile acid biosynthesis converting 7α,12α-dihydroxycholesterol (7α,12α-diHC), 7α,12α-dihydroxycholest-4-en-3-one (7α,12α-diHCO) and their reduced metabolite 5β-cholestan-3α,7α,12α-triol to their respective C_{27} carboxylic acids ready for side-chain shortening to ultimately yield cholic acid (Figure 1). CDCA can also be formed via the neutral pathway crossing over to the acidic pathway and also through reduction of 7α-hydroxycholest-4-en-3-one (7α-HCO) prior to C_{27} carboxylation by CYP27A1 and side-chain shortening. The pathology of CTX is often attributed to a build-up of toxic intermediates up-stream of CYP27A1 in the neutral pathway and, indeed, many of the clinical features of CTX can be resolved by treatment with CDCA if instituted early enough (6). However, CDCA does not appear to be effective in treating some of the neurological aspects of CTX especially if treatment is delayed and alternative strategies to restore the functional enzyme may have merit.

Figure 1. Abbreviated versions of the acidic and neutral pathways of bile acid biosynthesis. Metabolites absent or greatly diminished in plasma from CTX patients are indicated by a downward pointing thick red arrow, those elevated by an upward pointing thick blue arrow. The enzyme CYP27A1 is shown in red, other enzymes in blue. Defective reactions in CTX are shown by a crossed arrow. Cholesterol is drawn in red, its metabolites detected by positive-ion LC-ESI-MS^n following treatment with cholesterol oxidase and deuterium labelled Girard-P derivatization reagent [$^2H_5$]GP (Fr-1A) are in green, those detected following unlabelled [$^2H_0$]GP derivatisation in the absence of cholesterol oxidase treatment

(Fr-1B) are in claret, and those with a 5α-hydroxy plus carboxylic acid group detected by negative-ion LC-ESI-MS^n are shown in brown. A cholestanetetrol GlcA is shown in the inset with the GlcA drawn attached to C-3. **IMAGE IN FULL PAGE**.

CTX is an autosomal recessive genetic disorder, and in cases where pathogenic variants are present in both alleles of *CYP27A1* resulting in a largely inactive enzyme, diagnosis can be made by gene sequencing or by mass spectrometry (MS). This can be by gas-chromatography (GC)-MS detecting an elevation in cholestanol (5α-cholestane-3β-ol) in plasma or by detecting an increase in urinary or plasma bile alcohol glucuronides (GlcA) e.g. cholestanetetrol-, cholestanepentol-, cholestanehexol-GlcA, accompanied by a fall in CDCA by direct infusion (DI)-MS or liquid chromatography (LC)-MS (7-10). A disadvantage of cholestanol as a diagnostic biomarker of CTX is that it can be elevated in other genetic disorders like familial hypercholesterolemia and sitosterolaemia, as well as liver disease (10, 11). Diagnosis may be more difficult in cases where some enzyme activity is maintained, in which case it may be prudent to monitor additional metabolites such as 27-HC and 3β-HCA which will be almost absent when the enzyme is inactive but present at a reduced concentration where partial activity is maintained, or 7α-HCO and 7αH,12α-diHCO and bile alcohols which are highly elevated when CYP27A1 activity is deficient (12, 13).

Analytical Techniques to Detect CTX Biomarkers

GC-MS is applicable for measurement of cholestanol, 7α-hydroxycholesterol (7α-HC) and 7α-HCO, all of which are elevated in CTX plasma, and Lütjohann et al have recommended the latter two compounds as optimal markers in plasma for monitoring the response to therapy and their ratio to 27-HC, also measured in the same GC-MS run, as a good CTX diagnostic biomarker (10). While GC-MS is routinely used in dedicated mass spectrometry laboratories the additional requirement of derivatisation has, over the decades, led to a shift to more direct analysis methods. Historically elevated levels of bile alcohol glucuronides in urine or plasma/serum have also been used to diagnose CTX, first by fast atom bombardment (FAB)-MS and later by electrospray ionisation (ESI)-MS (7-9). Here there is no requirement for derivatisation, just a simple sample clean up, often by solid-phase extraction (SPE). Currently there is a move towards the use of ESI-tandem-MS (MS/MS or MS^2) in the screening for CTX from dried blood spots. Vaz et al have generated convincing data using ESI-MS/MS to demonstrate the ratio of cholestanetetrol-GlcA to taurochenodeoxycholic acid (TCDCA) as a screening biomarker for CTX, where cholestanetetrol-GlcA is elevated and TCDCA diminished (14). By also measuring the ratio of taurotrihydroxycholestanoic acid to cholestanetetrol-GlcA confusion with peroxisomal disorders can be avoided where cholestanetetrol-GlcA and taurotrihydroxycholestanoic acid are both elevated, but taurotrihydroxycholestanoic acid will be greatly diminished in CTX. Hong et al have added LC separation to the ESI-MS/MS diagnostic method, concluding that both cholestanetetrol-GlcA and the ratio of cholestanetetrol-GlcA to TCDCA are excellent CTX biomarkers suitable for newborn screening (15).

Measuring Multiple Bile Acid Precursors in CTX in a Single Analysis

The deficiency in CYP27A1 shifts cholesterol metabolism from the acidic to the neutral pathway; this is accentuated by a lack in production of CDCA resulting in reduced negative feedback via the farnesoid X receptor (FXR) on CYP7A1 expression, the first enzyme of the neutral pathway of bile acid biosynthesis (Figure 1), driving enhanced production of 7α-HC (8). Additionally, the block in the neutral pathway by deficient CYP27A1 results in an elevation in plasma of the early metabolites of the pathway i.e., 7α-HC, 7α-HCO, 7α,12α-diHC and 7α,12α-diHCO. These four metabolites fall into the family of molecules called oxysterols i.e. oxidised forms of cholesterol, which also include 27-HC, 7α,27-dihydroxycholesterol (7α,27-diHC), 7α,27-dihydroxycholest-4-en-3-one (7α,27-diHCO) and the

cholestenoic acids all of which are diminished in CTX (16-18). While these molecules can be detected by GC-MS it requires multiple different derivatisation steps, often with and without additional saponification necessary to hydrolyse sterol esters (19).

To optimise detection of these bile acid precursors we have developed a simple derivatisation strategy which allows the detection of most of the bile acid precursors in a single LC-ESI-$(MS)^n$ experiment (Figure 1) (20). In brief, oxysterols including cholestenoic acids, are extracted from plasma, into ethanol and after dilution with water passed through a C_{18} SPE column. Cholesterol and other highly lipophilic components are retained by the column leaving an eluent rich in oxysterols including cholestenoic acids. This eluate is divided into two equal fractions, Fr-1A and Fr1-B. Both fractions are dried down then reconstituted in propan-2-ol. To Fr-1A bacterial cholesterol oxidase enzyme in phosphate buffer is added. This converts sterols with a 3β-hydroxy group to equivalents 3-ones via the classical Richmond reaction (Figure 2A) (21, 22). [$^2H_5$]Girard P ([$^2H_5$]GP) reagent is then added in methanol, this reacts with the 3-oxo group to give a GP-hydrazone. The reaction mixture can be cleaned-up on a second reversed-phase SPE column to remove excess derivatisation reagent or injected directly onto a trap column preceding the LC column linked to the MS. The derivatised sterols give greatly improved ESI response and intense fragment ions useful for both quantification and identification (Figure 2) (16, 20, 23). However, some oxysterols and cholestenoic acids e.g. 7α-HCO, 7α,12α-diHCO, 7α-hydroxy-3-oxocholest-4-en-(25R/S)26-oic acid (7αH,3O-CA), naturally possess a native 3-oxo group and do not require an oxidation step prior to derivatisation. These can be analysed directly from Fr-1B which is treated as Fr-1A but in the absence of cholesterol oxidase and with the use of [$^2H_0$]GP instead of [$^2H_5$]GP (Figure 2B). The use of different isotopic GP reagents means that after the second SPE step equal aliquots of Fr-1A and Fr-1B can be combined and analysed in combination by LC-ESI-MS^n providing complementary information on cholesterol metabolites. Sterols derivatised with [$^2H_5$]GP can be differentiated from those derivatised with [$^2H_0$]GP by virtue of their difference in mass (5.0314 Da). This protocol allows the identification of all major cholesterol metabolites in the bile acid biosynthesis pathway with a 3β-hydroxy or 3-oxo group (Figure 1) and, with the inclusion of internal standards, absolute quantification (20).

Figure 2. Measuring multiple bile acid precursors via simple derivatisation and LC-ESI-MS^n. (A) Cholesterol metabolites with a 3β-hydroxy-5-ene structure are converted by bacterial cholesterol oxidase in the Richmond reaction to the equivalent 3-oxo-4-enes which are then derivatised with [$^2H_5$]GP (i.e. Fr-1A). (B) Metabolites with a native 3-oxo group are derivatised directly with [$^2H_0$]GP (i.e. Fr-1B). Fr-1A and Fr-1B are combined and analysed by LC-ESI-MS^n. Note, positive-negative ion switching allows the detection of bile acid precursor with a 3α-hydroxy group which are transparent to this derivatisation, providing they also have a carboxylic acid group. (C) Derivatised metabolites fragment by MS^2 to give abundant fragment-ions valuable for multiple-reaction monitoring and by MS^3 to give structural information. **IMAGE IN FULL PAGE.**

From data generated using this methodology the optimal CTX diagnostic biomarkers in plasma are the concentration ratios of 7α,12α-diHCO to 27-HC, 7α,12α-diHCO to 3β-HCA and 7α,12α-diHCO to 7αH,3O-CA (Figure 3) (16-18, 24). In each case there is no overlap in the measured ratios between control and CTX plasma and for the latter two ratios the controls differ from CTX by about three orders of magnitude. An advantage of the 7α,12α-diHCO to 7αH,3O-CA ratio is that both metabolites can be analysed in Fr-1B i.e. Fr-1A does not require preparation or analysis.

Figure 3. Concentration ratios of bile acid precursors in plasma provide unequivocal diagnosis of CTX. Ratios are shown on a log scale. Data from control samples are shown in blue (n = 24), from CTX patients on bile acid therapy are shown in red (n = 14), from untreated CTX patients in green (n = 4), and from CTX patients where treatment is unknown in purple (n = 4). In the case of 7αH,3O-CA

measurements were made from 12 CTX patients on bile acid therapy, 3 patients not on treatment, and one patient where the treatment regime was not known. [Data from Griffiths et al 2013 (16), Theofilopoulos et al 2014 (17), Abdel-Khalik et al 2017 (24) and Hoflinger et al 2021 (18)].

As discussed, LC-ESI-MS^n following GP-derivatisation allows the detection of all abundant bile acid precursors with a 3β-hydroxy-5-ene, 3β-hydroxy-5α-hydrogen or 3-oxo structure. This is performed in the positive-ion MS mode. However, primary bile acids and their immediate precursors possess a 3α-hydroxy group and are transparent to cholesterol oxidase treatment and GP-derivatisation. However, these metabolites often possess a carboxylic acid group or are conjugated with GlcA and can be readily analysed in the negative-ion MS mode. So, if we follow the cholesterol oxidase and GP-derivatisation protocol with positive-negative ion switching, bile acid pathway intermediates with a 3β-hydroxy, 3-oxo or 3α-hydroxy group can be detected providing the latter have also a carboxylic group. This covers almost the entire bile acid biosynthesis pathway plus a shunt to the bile alcohol glucuronides and cholestanol (Figure 1). For those cholesterol metabolites with a 3α-hydroxy group an alternative strategy is treatment with 3α-hydroxysteroid dehydrogenase to introduce a 3-oxo group for subsequent derivatisation with GP-reagent (25).

Unexpected Bile Acid Precursors Present in Plasma and CSF in CTX

CYP27A1 introduces the 27-hydroxy and 27-carboxylate groups onto the side-chain of cholesterol and other sterols leading to R-stereochemistry at the asymmetric centre at C-25. Other CYP enzymes can also hydroxylate the terminal carbon of sterols including CYP46A1 and CYP3A (26, 27) and we have found that CYP3A4 will 27-hydoxylate 7α-HC to 7α,27-diHC to specifically give S-stereochemistry at C-25, i.e. 7α,27-diHC(25S) (28).

When plasma or CSF is analysed from control individuals the cholestenoic acid 7αH,3O-CA is observed as both the 25R and 25S isomers, the latter being about 10 – 20% of the abundance of the former, with the two isomers being interconvertible via their CoA-thioesters by the enzyme alpha-methylacyl-CoA-racemase (AMACR) (4, 20). Interestingly, in CTX we also find both isomers at very low levels but of quite similar abundance (18, 20). This leads to the hypothesis that in CTX elevated 7α-HC can be hydroxylated by CYP3A4 to 7α,27-diHC(25S) which can the undergo oxidation by 3β-hydroxy-Δ^5-steroid oxidoreductase (HSD3B7) and further oxidation, perhaps by CYP3A4 or another oxygenase, at the terminal carbon to give 7αH,3O-CA(25S). This acid can undergo side-chain shortening and may account for the presence of low levels of CDCA sometimes observed in CTX. Alternatively, 7αH,3O-CA(25S) can isomerise to 7αH,3O-CA(25R) (4).

Bile Acid Precursors in Human Brain and their Potential Role in Pathophysiology of CTX

In human brain the major cholesterol metabolite is 24S-hydroxycholesterol (24S-HC), which can be exported from the brain and transported in the circulation to the liver for conversion to bile acids (29). Unlike cholesterol, oxysterols can cross the blood brain barrier; in this way 24S-HC is exported from, while 27-HC is imported into the brain. We have analysed brain tissue samples from a single CTX patient (18). Levels of 24S-HC were similar to controls, but 27-HC was absent in CTX while 7α-HCO and 7α,12α-diHCO were both elevated in CTX, as was cholestanol (18). Presumably both 7α-HCO and 7α,12α-diHCO enter the brain from the circulation as the necessary 7α- and 12α-hydroxylases (CYP7A1 and CYP8B1) are not expressed in brain (30). Interestingly, 7α,12α-diHCO and cholestanol were not found to be toxic to a neuronal-like cell line suggesting that some of the neurological problems associated with CTX may not be a consequence of toxic metabolites but rather an absence of neuroprotective metabolites (18, 31). In this regard, 27-HC, although toxic towards neurons at high concentration (32), may also be important to maintain cholesterol homeostasis in brain (18). 27-HC is

one of a number of side-chain oxysterols that binds to the endoplasmic reticulum anchor protein INSIG (insulin induced gene) causing a conformational change and the resultant binding of INSIG to the transport protein SCAP (SREBP cleavage activating protein), with the result that SCAP is unable to transport SREBP-2 (sterol regulatory element-binding protein-2), the master transcription for genes coding the enzymes of the cholesterol biosynthesis pathway and of the LDL-receptor, to the Golgi for processing to its active form (33). An absence of 27-HC could lead to a failure of this feedback mechanism and enhanced formation of cholesterol in brain and ultimately potentially toxic metabolites. In addition, 27-HC is a ligand to the liver X receptors (LXRs) (34), activation of which leads to transcription of the *ABCA1* and *APOE* genes coding for cholesterol transport and carrier proteins, respectively (35). Thus, an absence of 27-HC could enhance cholesterol synthesis in brain and also impair cholesterol export from brain cells, resulting in ineffective cholesterol homeostasis.

Besides being the enzyme that generates 27-HC from cholesterol, CYP27A1 is essential for the normal formation of the cholestenoic acids, e.g. 3β,7α-dihydroxycholest-5-en-(25R/S)-oic acid (3β,7α-diHCA) and 7αH,3O-CA. 7αH,3O-CA can be synthesised in brain and exported from brain. Although it is mostly considered as providing a route for removal of 27-HC from brain (36), it also offers a route for removal of cholesterol, its absence in CTX could also lead to ineffective cholesterol homeostasis. 3β,7α-diHCA is a precursor of 7αH,3O-CA and likewise is synthesised in brain. This acid has been shown to promote neuronal survival in an LXR-dependent fashion (17). 3β,7α-diHCA is a normal constituent of blood and its supplementation could provide a pharmacological treatment for CTX as it is likely to pass the blood brain barrier down a concentration gradient.

Considering the important functions of CYP27A1 in brain, an absence of its catalytic products may be more significant to the pathology of CTX, at least regarding the neurological aspects, than a build up of toxic bile acid intermediates (18). We speculate that a successful treatment approach may be pharmacological restoration of its enzyme products or replacement of defective CYP27A1. In principle this can be via enzyme replacement or adeno associated virus (AAV) vectors expressing *CYP27A1*. An AAV vector carrying *CYP46A1* is currently being used in a first in-human, phase 1/2 trial for the treatment of Huntington's disease. A word of warning regarding AAV-*CYP27A1* treatment and CTX. While the CYP27A1 metabolic product 3β,7α-diHCA is neuroprotective, other CYP27A1-derived metabolites, 27-HC, 3β-HCA and 3β,7β-diHCA can be neurotoxic (17), so a careful titration of enzyme would be required.

Conclusions

While genome sequencing can identify pathogenic variants in *CYP27A1*, sequencing often identifies variants of uncertain significance. Biochemical analysis can help to determine the resultant biochemical phenotype, and can be performed with comparative ease by currently available MS analytical methods to assist in providing a definitive diagnosis. Improved biochemical methods are likely to become ever more important with the uncovering of a broader clinical and biochemical phenotypic spectrum of disease for CTX than was previously recognized. In addition, MS methods provide an optimal approach to determine a deficiency in CYP27A1 activity in the realm of newborn screening, the next great opportunity for progress in improving the lives of CTX patients.

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Declaration of competing interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: WJG and YW are listed as inventors on the patent "Kit and method for quantitative detection of steroids" US9851368B2 and the patent application "Compound and method for the treatment and diagnosis of neurodegenerative conditions" US 2021/0139529 A1. WJG, EY and YW are shareholders in CholesteniX Ltd.

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