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PROGNOSTIC METHOD

5 The present invention relates to a new method of determining the functional activity of the MRP2 and/or MRP3 efflux pathway of a human or animal subject, and to the use of a bile acid derivative in such a method.

10 MRP2/Mrp2 (ABCC2/Abcc2), also known as Multidrug Resistance Protein 2, is a member of the ABCC subfamily of the ATP-Binding Cassette Transporter superfamily. An overview of the ATP-Binding Cassette transporter family is given in 'The Human ATP-Binding Cassette (ABC) Transporter Superfamily' by Michael Dean of the National Cancer Institute at Frederick, Maryland, published by the US National Center for Biotechnology Information. MRP2 is expressed on the apical
15 domain of hepatocytes, enterocytes of the proximal small intestine, and proximal renal tubular cells, as well as in the brain and the placenta.

MRP2 is important clinically as it modulates the pharmacokinetics of many drugs, and its expression and activity are also altered by a range of drugs and diseases.
20 For example, expression of MRP2 is altered in patients with liver diseases such as primary biliary cirrhosis, cholestasis (e.g. drug-induced cholestasis), fatty liver disease, alcoholic liver disease, primary sclerosing cholangitis, or viral hepatitis. In the kidney, MRP2 functions in renal elimination of substrates from the blood into urine. In the liver, MRP2 is the major exporter of organic anions from the liver into
25 the bile. MRP2 can transport sulphated and glucuronidated bile salts as well as other organic anions and/or their conjugates with glucuronate, glutathione and sulphate [Gerk et al J Pharmacol Exp Ther 2002 Aug; 302(2):407-415]. In addition to transport of conjugates, MRP2 transports a range of molecules including cancer chemotherapeutics, uricosurics, antibiotics, leukotrienes, glutathione, toxins and
30 heavy metals. MRP2 also plays an important role in the elimination of bilirubin glucuronosides from hepatocytes into bile. The MRP2 gene is mutated in patients with Dubin-Johnson syndrome, a human disorder of the organic ion transport. The absence of functional MRP2 from the canalicular membrane causes conjugated hyperbilirubinemia, as observed in the hereditary disorder Dubin-Johnson
35 syndrome.

Loss of MRP2 function is often well tolerated and compensated by the upregulation of other membrane transporters, particularly Multidrug Resistance Protein 3 (MRP3, also known as ABCC3) in the basolateral member of hepatocytes [Nies et al Pflugers Arch (2007) 453:643-659]. Like MRP2, MRP3 is a member of the ABCC subfamily of the ATP-binding cassette transporter superfamily. MRP3 is present in the intestine, kidney and the liver.

Knowledge of the state of the MRP2/3 activity of a subject therefore could be used to indicate a wide range of possible conditions.

It would therefore be advantageous to provide a method of determining the functional activity of the MRP2 and/or MRP3 efflux pathway of a human or animal subject.

We have unexpectedly found that MRP2 is the most prominent transporter responsible for biliary excretion of cholesteryl lysyl fluorescein (CLF), which is also known as fluorescein isocol, and that MRP3 is responsible for basolateral excretion of CLF. Accordingly, CLF and other bile acids having a similar metabolic/elimination pathway can be used in determining the functional activity of MRP2/3 efflux pathway.

The present invention is particularly surprising in view of the disclosure of Mills et al in Biochim Biophys Acta 1991 Dec 6;1115(2):151-156. They showed that, in rats, the biliary excretion rate of CLF after jugular vein injection has similar kinetics to that of glycocholate, the transport of which is known to be mediated by ABCB11. ABCB11 is a member of the ABCB subfamily of the ATP-binding cassette (ABC) transporter superfamily.

Other evidence that led to the belief that CLF is transported via ABCB11 was provided by Baxter et al [Biochim Biophys Acta 1995 Jun 6;1256(3):374-380]. Baxter et al administered glycocholate and CLF to isolated perfused rat livers under recycling conditions, and observed that CLF was capable of increasing phospholipids and cholesterol output in a similar way to that which was found for glycocholate. It was shown in the same study that rat liver has a much greater capacity to transfer glycocholate (GC) from perfusate to bile than CLF and

concomitantly, the increase in phospholipid and cholesterol output was less with CLF in comparison with GC.

5 In a later study [Mills et al in J Hepatol 1999 Oct;31(4):678-684], Mills et al investigated ABCC2/Abcc2 as a possible transporter for CLF. But they concluded from a study with normal and TR⁻ (*Abcc2* deficient) Wistar rats that *Abcc2* was not involved in the biliary excretion of CLF, based on the observation of similar biliary excretion in both strains.

10 In summary, prior to the present investigations, the totality of the prior art on the subject concluded unanimously that CLF was transported from the liver into bile by the ABCB11/Abcb11 pathway, rather than the ABCC2/Abcc2 pathway, and therefore would not have been considered as an agent for use in a method for the determination of MRP2/3 activity.

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According to the present invention, there is provided a method of determining the functional activity of the MRP2 and/or MRP3 efflux pathway of a human or animal subject, said method comprising:-

20 (i) determining the level of a bile acid derivative in the blood of said human or animal subject at a predetermined time interval after introducing an amount of the bile acid derivative into the subject, and

(ii) using the determination obtained in step (i) to indicate the functional activity of the MRP2 and/or MRP3 efflux pathway of the subject.

25 According to a further aspect of the invention there is provided a method of performing a prognosis or diagnosis on a human or animal subject, said method comprising:-

30 determining the level of a bile acid derivative in the blood of said human or animal subject at a predetermined time interval after introducing an amount of the bile acid derivative into the subject,

characterised in that the determination obtained is used to indicate the functional activity of the MRP2 and/or MRP3 efflux pathway of the subject.

35 The term "determining" in this context has a broad meaning. It includes both quantitative analysis to quantify the level or amount of the bile acid derivative in the sample and qualitative analysis to detect whether or not the bile acid is present and,

if appropriate, the degree to which it is present. Thus it is not essential for this method that a numerical value for the level of bile acid derivative in the blood is obtained. For example, ascertaining that the level of the bile acid derivative in the blood is within a certain range can be used to assess functional activity of the MRP2 and/or MRP3 pathway. By analogy, the words "determine" and "determination" are to be given a similar meaning.

The determination of the level of the bile acid can be carried out as described in European patent specification no. EP1,003,458 (the entire text of which is hereby imported by reference and which is intended to form an integral part of this disclosure). That specification describes the use of coloured or fluorescent bile acid derivatives in human subjects to determine the liver function of the subject. One example of such a bile acid derivative given in EP 1,003,458 is the fluorescent fluorescein derivative, cholyl lysyl fluorescein (CLF). EP 1,003,458 discloses the use of CLF as a potential liver function test.

The purpose of using a bile acid derivative compound, such as CLF, in a liver function test was to provide a compound which would be transported from hepatocytes into bile in the same way as natural bile acids. Bile acids are taken up into liver cells by at least two transport systems: a Na⁺ dependent system, involving Na⁺/taurocholate cotransporter polypeptide (known as NTCP) and a Na⁺ independent system, involving transporters in the organic anion transporter family (known as the OATP family). Bile acids are excreted into the bile ducts by the bile salt export pump, also known as BSEP or ABCB11, which expresses on the bile duct side of liver cells. As described above, prior to the present invention, it was believed that CLF is excreted into bile via ABCB11.

Instead, we have found that the above method can be used to indicate the functional activity of the MRP2 and/or MRP3 efflux pathway of a subject. MRP2/MRP3 expression and activity are altered by a wide range of drugs and disease states, therefore knowledge of the functional activity of the MRP2 and/or MRP3 elimination pathway of the subject can be used to indicate a wide range of conditions including those mentioned herein. This method is therefore useful in the diagnosis or prognosis of a range of conditions selected from those wherein MRP2 and/or MRP3 expression is modified, such as up-regulated or down-regulated, or wherein the

MRP2 and/or MRP3 gene is mutated. Preferably, the method of the present invention is used to diagnose or prognose a condition or disease specified herein.

5 The terms "functional activity of the MRP2 and/or MRP3 efflux pathway" or "functional activity of the MRP2 and/or MRP3 elimination pathway" as used herein refers to the transport activity of the MRP2 transporter protein and/or the MRP3 transporter protein in transporting substrates across cellular membranes.

10 The determination of the level of a bile acid derivative in the blood can be processed or analysed in a number of ways. The level of the bile acid derivative in the blood may be compared with at least one standard measurement. Similarly results taken at different predetermined time intervals may be compared with such standard measurements.

15 However, it is not necessary to make a comparison with a standard. It is possible to use the determination obtained to give a direct indication of the functional activity of the MRP2 and/or MRP3 elimination pathway of a subject, without the need for any comparison with results from a healthy subject or from a subject with known MRP2/3 function.

20 Preferably, the method comprises a further step of using the determination of the level of the bile acid derivative to arrive at a numerical value, wherein the numerical value indicates the functional activity of the MRP2 and/or MRP3 efflux pathway of the subject. A single numerical value indicating the functional activity of the MRP2
25 and/or MRP3 efflux pathway of the subject advantageously provides a useful means for physicians to assess functions of a patient that are affected by MRP2/3 activity, such as liver function, kidney function, or intestinal function. Said numerical value may be obtained by using the determination of the level of bile acid derivative to give a direct measure of the functional activity of the MRP2 and/or MRP3 efflux pathway
30 of the subject.

Preferably the determination obtained is compared with at least one standard to indicate the functional activity of the MRP2 and/or MRP3 efflux pathway of the subject.

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Preferably the level of the bile acid derivative in the blood of the subject is determined at two or more predetermined time intervals after introducing an amount of the bile acid derivative into the subject. By taking a series of blood samples over time, a plasma elimination curve may be constructed and compared with plasma elimination curves obtained from subjects having known MRP2/3 activity. Two or more determinations of level of the bile acid derivative in the blood of the subject taken at different time intervals may be used to obtain said numerical value indicating the functional activity of the MRP2 and/or MRP3 elimination pathway of the subject.

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For example, when the level of the bile acid derivative in the blood of the subject is determined at two or more predetermined time intervals, the determination obtained at a first time interval after administration and the determination obtained at a second time interval after administration can be expressed as a ratio or as a percentage. Thus a ratio of the determination after 30 minutes or after 60 minutes may be expressed as a ratio of the determination after 10 minutes. This ratio provides a numerical value, generally less than unity, which gives an indication of the functional activity of the MRP2 and/or MRP3 pathway of the subject.

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Alternatively, the determination at the later time interval, eg 30, 60 or 90 minutes, may be expressed as a percentage of the determination at the earlier time interval eg 10 minutes, following administration of the bile acid derivative. This percentage figure likewise gives an indication of the functional activity of the MRP2 and/or MRP3 pathway of the subject.

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Alternatively, a numerical value indicating the functional activity of the MRP2/3 pathway of a subject may be obtained by determining the slope of a log-linear plasma elimination curve derived from the determinations obtained. Alternatively, a numerical value may be obtained by determining the area under a plasma elimination curve derived from the results. Furthermore, the difference between the slopes of a log-linear plot derived from a test plasma elimination curve and a log-linear plot derived from a standard elimination curve can be calculated to provide said numerical value indicating the functional activity of the MRP2 and/or MRP3 pathway. Similarly, the difference between the areas under the plasma elimination curve derived from test results and a standard elimination curve can be calculated to provide said numerical value.

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The term "standard" in this context has a broad meaning. It is intended to encompass any single measurement or series (two or more) of measurements taken from a human or animal subject. This includes measurements taken from healthy
5 subjects, subjects with known MRP2/3 function, and can include a measurement or measurements taken from the subject whose MRP2/3 function is being investigated. That is to say, when one is concerned with the progression or staging of a disease one may compare measurements taken over time with the subject him(her)self acting as a control.

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Preferably the bile acid derivative comprises a marker such as a colour or a fluorescence.

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Preferably the method further comprises the step of providing at least one sample of blood which has passed through the liver of the subject and which sample has been collected at a predetermined time interval after administering the bile acid derivative to the subject, wherein the blood sample(s) are processed to obtain blood plasma or blood serum. Therefore, an *in vitro* method for determining the functional activity of the MRP2 and/or MRP3 efflux pathway of a subject may be provided.

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Preferably the blood sample(s) are processed by centrifugation. Preferably the blood proteins are separated from the blood plasma/blood serum.

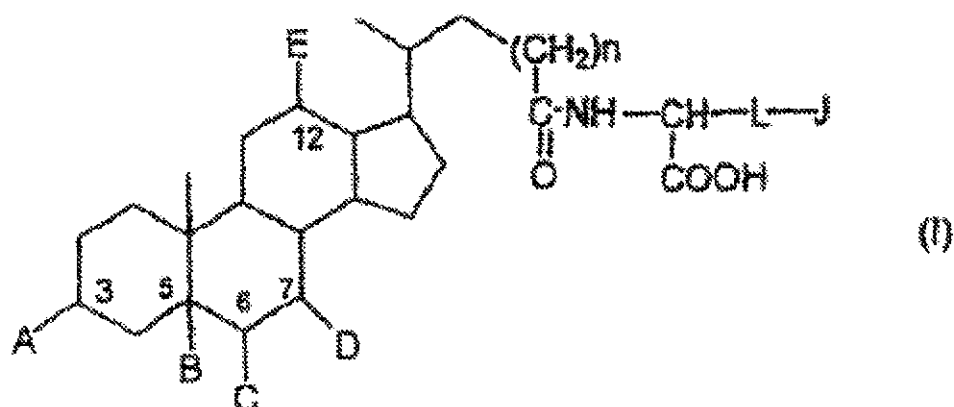
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Preferably the bile acid derivative is administered to the subject intravenously.

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Preferably the bile acid derivative comprises (a) a steroid moiety having (i) at least one substituent selected from the group consisting of a 3-hydroxyl substituent, a 7-hydroxyl substituent and a 12-hydroxyl substituent, and (ii) a carboxyl group attached by means of an amide linkage to a side chain of the steroid moiety; and (b) an active moiety which is to be targeted to the liver, said active moiety being attached to an α -carbon atom relative to the carboxyl group.

Particularly preferably the bile acid derivative has the general formula (I):-



wherein A is selected from the group consisting of α -OH and β -OH; B is selected from the group consisting of α -H and β -H; C is selected from the group consisting of -H, α -OH and β -OH; or B and C together form a double bond; D is selected from the group consisting of -H, α -OH and β -OH; E is selected from the group consisting of -H, α -OH and β -OH; L is a linking moiety; J is a coloured or fluorescent moiety; and n is 0 or 1.

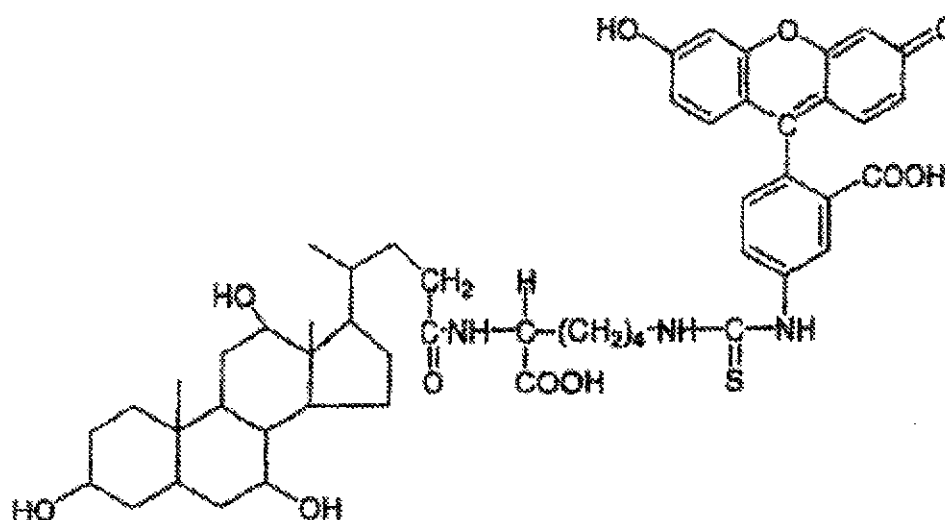
In the formula above the linking moiety, L, is preferably N-terminated at its end attached to the coloured or fluorescent moiety, J, and is preferably $-(CH_2)_nNH$, where n is 3 or 4, $-(CH_2)_4NH-(CH_2)_3NHC(=NH)NH-$, or $-(CH_2)_2-CH(OH)CH_2NH-$.

Alternatively, the moiety $-NH-CH(COOH)-L-$ may be derived from S-adenosylhomocysteine, S-adenosylmethionine, S-amino-imadazole-4- carboxamide, asparagine, cadaverine, cystamine, citrulline, diaminopimelic acid, 2,4-diaminobutyric acid, cysteamine, glutamine, 3-hydroxykynurenine, kynurenine, putrescine or negamycin. Alternatively, acidic amino acids can be used instead of the above where active moiety J has one amino group and/or is hydrophobic.

In the formula above preferably J is or includes a fluorescein, rhodamine or other fluorescing moiety.

Preferably the steroid moiety of the bile acid derivative is based on an acid selected from the group consisting of cholic acid, chenodeoxycholic acid, deoxycholic acid, hyodeoxycholic acid, hyocholic acid, α -, β - or ω -muricholic acid, nor-bile acids, lithocholic acid, 3 β -hydroxycholenoic acid, ursodeoxycholic acid and allocholic acid (5 α -cholan-24-oic-acid).

Most preferably the bile acid derivative is a cholyI-lysyl-fluorescein (CLF) having the formula:-



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or salts thereof. Preferably the bile acid derivative is a trisodium salt. Preferably the bile acid derivative is cholyI-L-lysyl-fluorescein, also known as fluorescein lisicol. Preferably the bile acid derivative is fluorescein lisicol trisodium salt.

10

According to a further aspect of the invention there is provided use of MRP2 and/or MRP3 functional activity obtained from the above method for determining the liver function of a human or animal subject.

15 According to a further aspect of the invention there is provided use of a bile acid derivative for determining the functional activity of the MRP2 and/or MRP3 efflux pathway of a human or animal subject.

20 According to a further aspect of the invention there is provided use of a bile acid derivative in the diagnosis or prognosis of Dubin-Johnson syndrome, cirrhosis of the liver, cholestasis, fatty liver disease, alcoholic liver disease, primary sclerosing cholangitis, or viral hepatitis.

25 According to a further aspect of the invention there is provided use of a bile acid derivative in the manufacture of a diagnostic or prognostic agent for use in the

diagnosis or prognosis of Dubin-Johnson syndrome, cirrhosis of the liver, cholestasis, fatty liver disease, alcoholic liver disease, primary sclerosing cholangitis, or viral hepatitis.

- 5 According to a further aspect of the invention there is provided use of a bile acid derivative in determining the state of the bilirubin clearance pathway or conjugated bilirubin clearance pathway of a human or animal subject.

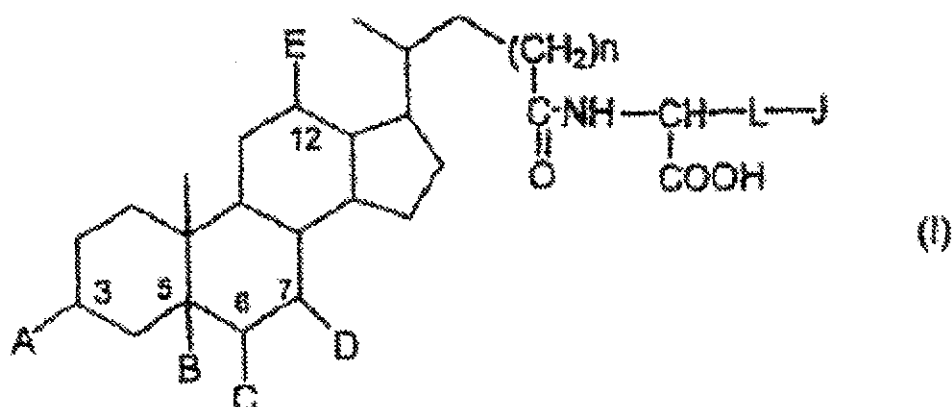
- 10 According to a further aspect of the invention there is provided use of a bile acid derivative in the manufacture of a diagnostic or prognostic agent for determining the state of the bilirubin clearance pathway or conjugated bilirubin clearance pathway of a human or animal subject.

- 15 According to a further aspect of the invention there is provided use of a bile acid derivative for determining the kidney function, biliary function, intestinal function, or liver function of a human or animal subject.

- 20 According to a further aspect of the invention there is provided use of a bile acid derivative in the manufacture of a diagnostic or prognostic agent for determining the kidney function, biliary function, intestinal function, or liver function of a human or animal subject.

- 25 Preferably said bile acid derivative comprises (a) a steroid moiety having (i) at least one substituent selected from the group consisting of a 3-hydroxyl substituent, a 7-hydroxyl substituent and a 12-hydroxyl substituent, and (ii) a carboxyl group attached by means of an amide linkage to a side chain of the steroid moiety; and (b) an active moiety which is to be targeted to the liver, said active moiety being attached to an α -carbon atom relative to the carboxyl group.

- 30 Preferably said bile acid derivative is a compound of formula (I)



wherein A is selected from the group consisting of α -OH and β -OH; B is selected from the group consisting of α -H and β -H; C is selected from the group consisting of -H, α -OH and β -OH; or B and C together form a double bond; D is selected from the group consisting of -H, α -OH and β -OH; E is selected from the group consisting of -H, α -OH and β -OH; L is a linking moiety; J is a coloured or fluorescent moiety; and n is 0 or 1.

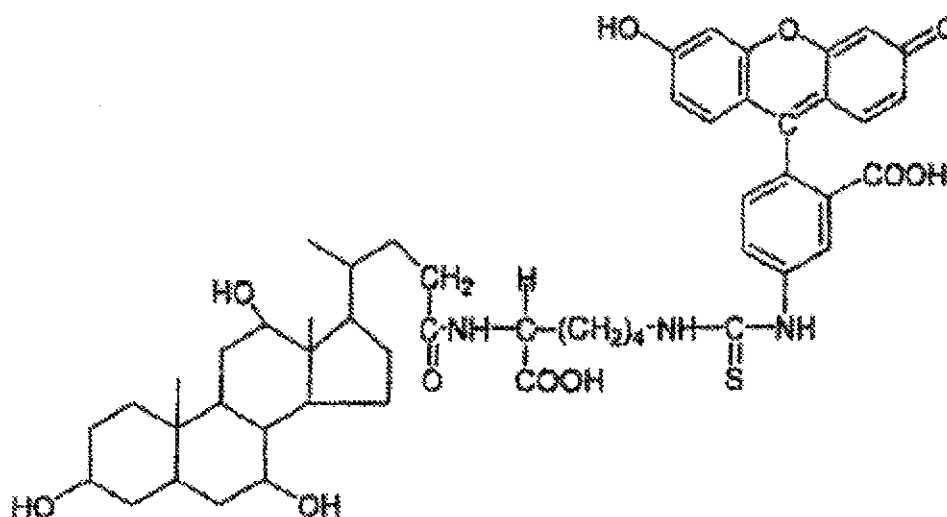
Preferably J is or includes a fluorescein, rhodamine or other fluorescing moiety.

Preferably the linking moiety is N-terminated at its end attached to active moiety J, and L is selected from the group consisting of: $-(CH_2)_nNH$, where n is 3 or 4, $-(CH_2)_4NH-(CH_2)_3NHC(=NH)NH-$, and $-(CH_2)_2-CH(OH)CH_2NH-$.

Preferably the moiety $-NH-CH(COOH)-L-$ in the general formula (I) is derived from a compound selected from the group consisting of S-adenosylhomocysteine, S-adenosylmethiodine, S-aminoimidazole-4-carboxamide, asparagine, cadaverine, cystamine, citrulline, diaminopimelic acid, 2,4-diaminobutyric acid, cysteamine, glutamine, 3-hydroxykynurenine, kynurenine, putrescine and negamycin.

Preferably the steroid moiety of the bile acid derivative is based on an acid selected from the group consisting of cholic acid, chenodeoxycholic acid, deoxycholic acid, hyodeoxycholic acid, hyocholic acid, α -, β - or ω -muricholic acid, nor-bile acids, lithocholic acid, 3 β -hydroxycholenoic acid, ursodeoxycholic acid and allocholic acid (5 α -cholan-24-oic-acid).

Preferably the bile acid derivative is a cholyl-lysyl-fluorescein (CLF) having the formula:-



or salts thereof. Preferably the bile acid derivative is a trisodium salt. Preferably the
5 bile acid derivative is fluorescein lisicol trisodium salt.

The Applicant has unexpectedly found that, contrary to the teaching of the prior art, MRP2 and MRP3 transport compounds of general formula (I). A compound of said
10 formula can therefore be used for determining the functional activity of the MRP2 and/or MRP3 pathway of a human or animal subject.

The compounds of formula (I) may be used in the diagnosis or prognosis of Dubin-Johnson syndrome or in the manufacture of a diagnostic or prognostic agent for use
15 in the diagnosis or prognosis of Dubin-Johnson syndrome. Dubin-Johnson syndrome is linked to mutations in the MRP2 gene. Accordingly determination of the activity MRP2 and/or MRP3 pathway of a subject can provide information for use in diagnosis or prognosis relating to Dubin-Johnson syndrome.

The compounds of formula (I) may also be used in determining the kidney function, biliary function, intestinal function, or liver function of a human or animal subject, or
20 used in the manufacture of a diagnostic or prognostic agent for determining the kidney function, biliary function, intestinal function, or liver function of a human or animal subject. MRP2 is expressed in the kidney, intestine and liver, therefore determination of the activity MRP2 and/or MRP3 pathway of a subject can provide

information for use in determining kidney function, biliary function, intestinal function, or liver function of a subject.

5 The compounds of formula (I) may also be used in diagnosis or prognosis of cirrhosis of the liver, cholestasis, fatty liver disease, alcoholic liver disease, primary sclerosing cholangitis, or viral hepatitis, or in the manufacture of a diagnostic or prognostic agent for use in the diagnosis or prognosis of cirrhosis of the liver, cholestasis, fatty liver disease, alcoholic liver disease, primary sclerosing cholangitis, or viral hepatitis.

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The compounds of formula (I) may also be used in determining the state of the bilirubin clearance pathway or conjugated bilirubin clearance pathway of a human or animal subject, or in the manufacture of a diagnostic or prognostic agent for determining the state of the bilirubin clearance pathway or conjugated bilirubin clearance pathway of a human or animal subject.

15

Bilirubin is a breakdown product of heme catabolism. In the liver, bilirubin is conjugated with glucuronic acid and is excreted in bile. Bilirubin and conjugates of bilirubin, including as bilirubin glucuronides such as glucuronosyl-bilirubin, are transported via MRP2 [Jedlitschky et al, Expert Opin Drug Metab Toxicol 2006 Jun 20 2(3):351-66]. Increased levels of conjugated bilirubin in plasma indicate a possible disorder in biliary transport of conjugated bilirubin, hence serum bilirubin levels are useful in predicting mortality of liver transplantation candidates.

25 Bilirubin and bilirubin conjugates are transported via the MRP2 efflux pathway, therefore determinations of the functional activity of the MRP2 and/or MRP3 efflux pathway can be used to determine clearance activity of bilirubin or conjugated bilirubin. Determination of bilirubin and conjugated bilirubin clearance can give an indication of the state of the bilirubin efflux pathway. By determining the state of the bilirubin clearance pathway or conjugated bilirubin clearance pathway of a subject, this can indicate disorders in bilirubin or conjugated bilirubin transport.

30

The terms "bilirubin clearance pathway" and "conjugated bilirubin clearance pathway" as used herein refer to the transport of bilirubin/ conjugated bilirubin across cellular membranes.

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The preferred features of these aspects are as described above.

According to a further aspect of the invention, there is provided a computer program for determining the functional activity of the MRP2 and/or MRP3 elimination pathway
5 of a human or animal subject by using a determination of the level of a bile acid derivative in the blood of a human or animal subject at a predetermined time interval after introducing an amount of the bile acid derivative into the subject to arrive at a numerical value, wherein the numerical value indicates the functional activity of the MRP2 and/or MRP3 elimination pathway of the subject.

10

According to a further aspect of the invention, there is provided a method of determining the functional activity of the OATP1B3 uptake pathway of a human or animal subject, said method comprising:-

(i) determining the level of a bile acid derivative in the blood of said human or
15 animal subject at a predetermined time interval after introducing an amount of the bile acid derivative into the subject; and

(ii) using the determination obtained in step (i) to indicate the functional activity of the OATP1B3 uptake pathway of the subject.

20 Previous studies showed partial sodium-dependent uptake of some, but not all, fluorescent bile salts into rat hepatocytes [Maglova LM et al, Hepatology 1995 Aug;22(2):637-647]. In addition, uptake of cholyl-glycyl-fluorescein into CHO cells expressing rat Ntcp, but not wild type cells, has been demonstrated [Boyer JL et al, Am J Physiol 1994 Mar;266(3 Pt 1):G382-G387]. It could therefore be expected that
25 CLF may be taken up into hepatocytes via NTCP. Instead, we have found that CLF is taken up into hepatocytes via OATP1B3, and we have found that the above method can be used to indicate the functional activity of the OATP1B3 uptake pathway of a subject.

30 The preferred features of this aspect of the invention are as described above in relation to the previously described aspects of the invention.

The invention will now be described by way of example with reference to the drawings, in which:-

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Fig. 1A is an immunofluorescence micrograph showing expression of Na⁺/taurocholate cotransporter polypeptide (NTCP) in stably transfected cells. In Fig. 1A, the fluorescent parts of the micrograph appear dark and the non-fluorescent parts appear white. NTCP is a protein that mediates uptake of bile acids into liver cells. NTCP was visualised as described in the material and methods section below.

Fig. 1B shows kinetic characterisation of CHO-NTCP wherein cells were incubated with increasing TC (taurocholate) concentrations for 45 seconds in the presence of sodium (■) or potassium (▲). Sodium-dependent uptake rates (▼) were used to determine the kinetic parameters. Data represent means±SD of triplicates. Where error bars are absent, they were smaller than the symbol.

Figures 2A – 2D relate to uptake studies on CHO cells expressing hepatocellular uptake transporters; the data for Fig. 2B – 2D represent means±SD.

Fig. 2A shows fluorescence pictures after incubation of CHO transporter cells with 1µmol/L CLF over 5 minutes. Fluorescent pictures (right panels) are complemented with phase contrast pictures (left panels). In the fluorescence pictures (right panels), the fluorescent parts appear dark and the non-fluorescent parts appear white.

Fig. 2B is a graph showing transport of CLF mediated by NTCP in the presence (black bars) and absence of sodium (white bars) for 1 and 5 minutes.

Fig. 2C is a graph showing transport of CLF for CHO cells (wild type and cells transfected with OATP1B1, OATP1B3 and OATP2B1). Cells were incubated with 1µmol/L CLF during 1 (white bar) or 5 minutes (black bars).

Fig. 2D relates to kinetics of OATP1B3 mediated CLF transport and shows a graph of CLF transport against CLF concentration. Cells were incubated with increasing concentrations of CLF for 45 seconds. Shown are uptake rates corrected for 0seconds binding.

Figs. 3A – 3D relate to inhibition of transport activity of hepatocellular uptake transporters by CLF. CHO cells expressing NTCP, OATP1B1, OATP1B3 and OATP2B1 were incubated with 0, 5 or 50µmol/L CLF and with the substrates,

2.5 μ mol/L TC, 1 μ mol/L estrone-3-sulfate, 10 μ mol/L TC and 1 μ mol/L estrone-3-sulfate, respectively. Data represent means $\pm$ SD of triplicate determinations.

Fig. 3A shows the results for CHO cells expressing NTCP with 2.5 μ mol/L TC.

5 Fig. 3B shows the results for CHO cells expressing OATP1B1 with 1 μ mol/L estrone-3-sulfate.

Fig. 3C shows the results for CHO cells expressing OATP1B3 with 10 μ mol/L TC.

Fig. 3D shows the results for CHO cells expressing OATP2B1 with 1 μ mol/L estrone-3-sulfate.

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Fig. 4A shows Western blots of membrane vesicles of Sf21 insect cells uninfected and infected with ABCB11, ABCC2, ABCC3 and ABCG2 cDNA containing baculovirus. The Western blots show the presence of proteins ABCB11, ABCC2, ABCC3 and ABCG2 in the respective infected Sf21 insect cells.

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Figs. 4B to 4F show time-dependent transport of CLF into plasma membrane vesicles from wild type Sf21 cells (B), Sf21 cells expressing ABCB11 (C), ABCG2 (D), ABCC2 (E) or ABCC3 (F), respectively.

20 Figs. 4G and 4H show concentration-dependent uptake of CLF into Sf21 membrane vesicles containing ABCC2 (G) or ABCC3 (H) respectively.

25 Figs. 5A and 5B show CLF inhibition of DNP-SG and TC transport into ABCC2 (A) and ABCB11 (B) protein containing Sf21 membrane vesicles respectively. The values are expressed as a percentage of maximal ATP-dependent transport. Significance was tested using two-sided Student's t-test: *P<0.05 for transport in the presence vs. absence of inhibitor.

30 Fig. 6 shows plasma clearance of CLF in mice. Wild type, *Abcc3*^{-/-} and *Abcc2*^{-/-} mice received 100 μ L of 2mmol/L CLF by injection in the tail vein. After the indicated time points blood was drawn. Data are expressed as the mean percentage of the initial CLF level at 2minutes. Significance was tested using two-sided Student's t-test: *P<0.05 for *Abcc2*^{-/-} vs wild type mice.

35 Fig. 7A shows time dependent appearance of CLF in bile of wild type, *Abcc3*^{-/-} and *Abcc2*^{-/-} mice. Data represent means $\pm$ SD.

Fig. 7B shows CLF levels in blood, urine, liver and bile of wild type, *Abcc3*^{-/-} and *Abcc2*^{-/-} mice after 2 hours. Mice received 100 μ L of 1 mmol/L CLF by injection in the tail vein. Data represent means $\pm$ SD. Plasma levels are expressed in nmol/L and levels in urine, liver and bile as percentage of administered dose. Significance was tested using two-sided Student's t-test: * $P < 0.05$ for *Abcc2*^{-/-} vs wild type mice. Data represent means $\pm$ SD.

Fig. 8 relates to intestinal uptake of TC and CLF. Appearance of TC and CLF in bile after ileal administration of 100 μ L mixture of 2 mmol/L TC and CLF in wild type mice. Bile was collected after indicated time points. Data represents means $\pm$ SD. The graph is a plot of cumulative biliary levels as percentage of applied dose. Significance was tested using two-sided Student's t-test: * $P < 0.05$ for TC uptake vs CLF uptake.

Fig. 9 shows plasma elimination curves relating to Example 2. The left hand graph has a linear and the right hand graph has a log-linear axis.

Fig. 10 shows plasma elimination curves relating to Example 10. The left hand graph has a linear and the right hand graph has a log-linear axis.

Example 1: Demonstration that the Bile Acid Derivative CLF is a Substrate for MRP2

Materials

Cholyl lysyl fluorescein (CLF) was obtained from Norgine, Harefield, United Kingdom. [³H]GSH (52 Ci/mmol) and [³H]TC (1.19 Ci/mmol) were obtained from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). [³H]DNP-SG synthesis was performed as described by de Waart DR et al [Liver Int 2006 Apr;26(3):362-368]. [³H]E₂17 β G (40.5 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, USA). Cellulose acetate membrane filters were bought from Schleicher and Schuell (Dassel, Germany). Sucrose was obtained from Brunschwig Chemie (Amsterdam, The Netherlands). Creatine phosphate was purchased from Boehringer Mannheim (Almere, The Netherlands). Creatine kinase was bought from Roche Diagnostics (Mannheim, Germany). Methanol (HPLC grade) was obtained from Baker (Deventer, The Netherlands). Triton X-100 was

bought from Merck (Darmstadt, Germany). All other chemicals and reagents were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Animals

- 5 *Abcc2*^{-/-} mice (against a Friend virus B-type (FVB) background) were bred at the National Cancer Institute (Amsterdam, The Netherlands). Production and characterisation of the *Abcc2*^{-/-} mice was as described by Vlamings *et al* [J Pharmacol Exp Ther 2006 Jul;318(1):319-327]. Wild type and *Abcc3*^{-/-} mice (against an FVB background) were bred at the animal institute of the Academic Medical
10 Center. Production and characterisation of the *Abcc3*^{-/-} mice was as described by Zelcer *et al* [J Hepatol 2006 Apr;44(4):768-775].

Generation of CHO cells stably expressing NTCP and Cell Lines

- cDNA for human NTCP [Hagenbuch *et al*, J Clin Invest 1994 Mar;93(3):1326-1331]
15 was cut with *EcoRV* and *HindI* and the coding region ligated into pcDNA5/FRT (Invitrogen, Life-Technologies, Carlsbad, CA). CHO FlpIn cells (Invitrogen, Life-Technologies) were transfected with the resulting construct using Lipofectamine[®] 2000 (Invitrogen, Life-Technologies). Stably transfected cells were selected with 550µg Hygromycin-B in HAM-F12 medium (Gibco, Invitrogen, Life-Technologies).
20 Transfected cells were cloned with the limiting dilution method. Clones expressing functional NTCP were identified by transport assays with radioactively labelled taurocholate (see below). To further characterise the cloned cells, immunofluorescence localization of NTCP using a polyclonal antibody against NTCP [Kullak-Ublick G *et al*, Gastroenterology 1997 Oct;113(4):1295-1305] was performed
25 as described by Huber RD *et al* [Am J Physiol Cell Physiol 2007 Feb;292(2):C795-C806]. CHO cells expressing OATP1B1, OATP1B3 and OATP2B1 were described previously [Treiber *et al*, DMD 35:1400-1407, 2007].

Transport experiments with stably transfected cell lines

- 30 For all transport experiments, cells grown on 3cm culture dishes were cultured for 24 hours in media supplemented with 5mmol/L sodium-butyrate to increase expression levels of transfected transporters [Palermo DP *et al*, J Biotechnol 1991 Jun;19(1):35-47].

- Transport experiments with uptake of radioactively labelled substrates: These experiments were performed as described by Huber RD et al [Am J Physiol Cell Physiol 2007 Feb;292(2):C795-C806].

5 - Transport experiments with uptake of CLF: Uptake was performed in the same buffers as for the radioactive substrates above. For visualization of CLF uptake, cells were immediately inspected with a Leica-DM-IRBE-inverted-microscope (Leica-Microsystems,Wetzlar,Germany) equipped with a Hamamatsu-ORKA-ER-camera (Hamamatsu-Photonics,Japan). To determine uptake of CLF, cells were
10 solubilized by the addition of 2ml 1% (w/v) Triton-X-100. After complete solubilization, 1.5ml was used to measure fluorescence in a Perkin-Elmer LS-5 luminescence-spectrometer set at λ_{exc} 486nm (slit 10nm) and λ_{em} 520nm (slit 5nm). Protein was determined with the bicinchoninic acid method using a kit from Interchim (Montfaucon,France). Transport data from OATP expressing cell lines
15 were corrected for binding by subtracting 0min time points (blank values) and except for figs. 2B and 2C were normalised per minute. Kinetic analysis was performed with nonlinear regression of the data to the Michaelis-Menten equation using GraphPad PRISM-V-4.00 (GraphPad-Software-Inc.,San Diego,CA)

20 Preparation of Membrane Vesicles

The production of recombinant baculovirus was as described by de Waart et al [Liver Int 2006 Apr;26(3):362-368]. *ABCC3* and *ABCG2*-recombinant baculovirus were a kind gift from Borst [Breedveld P. et al, Cancer Res 2004 Aug 15;64(16):5804-5811 and Borst P. et al, J Biol Chem 2001 Dec 7;276(49):46400-
25 46407.]. *ABCB11*-recombinant baculovirus was a kind gift from Thompson [Thompson RJ et al, Gastroenterology 2002 Nov;123(5):1649-1658]. *Sf21* cells grown at 27°C were infected with *ABCB11*, *ABCC2*, *ABCC3* and *ABCG2*-cDNA containing baculovirus. Cells were harvested at 2-4 days after infection. Membrane vesicle preparation was as described by de Waart et al [Liver Int 2006
30 Apr;26(3):362-368].

Western blotting and protein analysis

Membrane vesicles were fractionated by 6% SDS-PAGE, blotted on nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany) which were blocked in
35 phosphate-buffered saline (PBS)/5% milk powder/0.05% Tween-20. The following antibodies were used: anti-his probe; sc-803 (Santa Cruz, USA), anti-ABCG2; BXP-

21 [Malliepaard M et al, Cancer Res 2001 Apr 15;61(8):3458-3464], anti-ABCC2;
 M₂III6 [Scheffer et al, Cancer Res 2000 Sep 15;60(18):5269-5277] and anti-ABCC3;
 M₃III21 [Scheffer et al, Cancer Res 2000 Sep 15;60(18):5269-5277]. Immune
 complexes were visualized with horseradish-peroxidase-conjugated
 5 immunoglobulins and detected using chemiluminescence (Amersham, UK).

Transport assays with plasma membrane vesicles

Transport studies with membrane vesicles were performed using the rapid filtration
 technique as described by Heijn M et al [Am J Physiol 1992 Jan;262(1 Pt 1):C104-
 10 C110]. Radiolabel was measured with a scintillation counter. When CLF was used
 as a probe, filters were placed in a glass tube, 0.1% Triton-X-100 was added and
 the tubes vortexed. Samples were pipetted in 96-well plates (Kartell, Noviglio, Italy)
 and the amount CLF quantitated by measurement of fluorescence at λ_{exc} 485nm and
 λ_{em} 520nm using a NovoStar (BMG-labtech, Offenburg, Germany).

15

Animal experiments

Male mice were housed in a pathogen-free animal facility on a 12 hours light-dark
 cycle. Mice were anesthetized with a combination of Hypnorm (VetaPharma, UK;
 11.8mg/kg fluanisone and 0.37mg/kg fentanyl-citrate) and diazepam
 20 (Centrafarm, Etten-Leur, The Netherlands; 5.9mg/kg valium). Body temperature was
 maintained at 36±1°C on thermostatted heating pads. For clearance studies, mice
 were infused with CLF by injecting 100µL (2mmol/L) CLF in the tail vein.
 Subsequently, blood was drawn from the carotis at indicated time points. Blood
 samples were deproteinised by addition of two volumes methanol and the amount
 25 CLF in the supernatant was quantitated by measurement of fluorescence as
 described above.

For biliary secretion studies, the gall bladder was cannulated with PE10
 polyethylene tubing and 100µL (1mmol/L) CLF was injected in the tail vein. Bile was
 30 collected in 10 minutes fractions; liver and blood were harvested at the end of the
 experiment. Homogenised livers were deproteinised by adding 2 volumes of MeOH,
 and bile and blood samples were diluted with 0.1% Triton-X-100. The amount CLF
 was quantitated by measurement of fluorescence as described above.

35 For intestinal uptake studies of taurocholate (TC) and CLF, mice were anesthetised
 and the gall bladder cannulated as described above. Mice received both TC and

CLF by injecting 100 μ L (2mmol/L) CLF into the ileum. Bile was collected every 15 minutes. Radioactivity was measured in a scintillation counter and CLF quantitated as described above.

5 Results

The role of different hepatocellular bile salt transporters in the hepatic uptake of CLF was investigated. For this purpose CHO cells stably expressing NTCP were generated (CHO-NTCP) and characterised. Figure 1A shows an immunofluorescence micrograph of stably transfected cells from an
 10 immunofluorescence assay using a polyclonal antibody against NTCP. An immunofluorescence micrograph for wild type cells (not shown) displayed no fluorescence and therefore no expression of NTCP in wild type CHO cells, but the immunofluorescence micrograph for transfected cells (Fig 1A) shows fluorescence at the cell membranes and therefore shows clear plasma membrane expression of
 15 NTCP in stably transfected cells. Furthermore, uptake of taurocholate (TC) into these cells was sodium dependent (data not shown) and was saturable with increasing taurocholate concentrations. Even at high taurocholate concentrations, uptake in the absence of sodium was negligible (Fig 1B). The K_m and the V_{max} values were 16.0 μ mol/L and 6738pmol TC/mg protein/min, respectively, for sodium-dependent transport of TC (Fig 1B). On the contrary, no sodium-dependent uptake
 20 of CLF mediated by NTCP could be observed by fluorescence analysis and by quantitative determination of CLF of NTCP cells incubated with CLF (Fig 2A and 2B, respectively). The fluorescence picture of the CHO cells expressing NTCP (see Fig 2A) shows absence of CLF uptake into cells. The long cell towards the right of the picture of CHO cells expressing NTCP, which is exhibiting weak fluorescent
 25 staining, may not have been vital anymore. The uptake of CLF in the absence of sodium (Fig 2B) was comparable to that of wild type cells (not shown) and tended to increase minimally with increasing incubation time.

30 Next, uptake of CLF mediated by OATP1B1, OATP1B3 and OATP2B1 was examined in wild type and stably transfected CHO cells. The fluorescence pictures of the CHO cells expressing OATP1B1, OATP1B3 and OATP2B1 (see Fig 2A) show uptake of CLF for the CHO cells expressing OATP1B3 but not for OATP1B1 and OATP2B1. The fluorescence picture of the CHO cells expressing OATP1B3 shows
 35 strong fluorescent staining of the cells, and therefore shows clear uptake of CLF into the OATP1B3 expressing cells. The fluorescence picture of the CHO cells

expressing OATP1B1 shows very weak fluorescent staining, showing that uptake of CLF into OATP1B1 cells was very weak but consistent. In contrast, no uptake into OATP2B1 was found, the fluorescence picture of the CHO cells expressing OATP2B1 showing no fluorescent staining except for a single positive cell visible in the fluorescence picture, which may be a cell with partially reduced vitality, as judged by the phase contrast microscopy. High, time dependent transport rates of CLF were only seen in OATP1B3 expressing CHO cells (Fig. 2A and 2C, respectively). Uptake of CLF by OATP1B1 cells was consistently observed, but not by OATP2B1 cells (Fig 2A and 2C), indicating that CLF is not a substrate for OATP2B1. OATP1B3 mediated CLF transport was concentration-dependent (Fig 2D) with K_m and the V_{max} values of $4.6 \pm 2.7 \mu\text{mol/L}$ and $213 \pm 42 \text{ pmol CLF/mg protein/min}$, respectively (mean of three independent determinations). Uptake experiments with increasing CLF concentrations and OATP1B1 cells showed evidence for saturability (not shown). Due to a low signal to noise ratio, in particular at higher CLF concentrations, no determination of the kinetic parameters was possible.

Investigations were carried out to determine whether transport of model compounds mediated by NTCP, OATP1B1, OATP1B3 and OATP2B1 could be inhibited by CLF despite CLF being a substrate for OATP1B3 only. CHO cells expressing NTCP, OATP1B1, OATP1B3 and OATP2B1 were incubated with 0, 5 or $50 \mu\text{mol/L}$ CLF and with the substrates, $2.5 \mu\text{mol/L}$ TC, $1 \mu\text{mol/L}$ estrone-3-sulfate, $10 \mu\text{mol/L}$ TC and $1 \mu\text{mol/L}$ estrone-3-sulfate, respectively. Data represent means $\pm$ SD of triplicate determinations. Strikingly, CLF very efficiently inhibited the transport of TC via NTCP (Fig 3A). Transport of TC mediated by OATP1B3 could be inhibited by CLF in a dose-dependent manner (Fig 3C) as expected. Finally, transport of the model substrate estrone-3-sulfate mediated by OATP1B1 and OATP2B1 was inhibited by CLF, albeit much less efficiently (Fig 3B and 3D respectively), corroborating the uptake experiments with CLF. Of note, addition of CLF to wild type cells had no influence on substrate uptake indicating that it does not interfere with the structural integrity of the plasma membrane (Fig. 3).

In order to test whether the canalicular ATP-dependent transporters ABCB11, ABCC2 and ABCG2, as well as the basolateral transporter ABCC3, mediate transport of CLF, these human proteins were expressed in Sf21 insect cells (Fig 4A). Figure 4B shows that slight ATP-dependent uptake occurred in control

vesicles, indicating that an endogenous transporter is able to take up CLF in an ATP-dependent fashion. ATP-dependent transport of CLF in ABCB11 and ABCG2 protein containing membrane vesicles was not higher than in control vesicles (Fig 4C and D, respectively). However, ABCC2 and ABCC3 containing membrane vesicles showed much higher CLF transport rates compared to control wild type Sf21 membrane vesicles (Fig 4E and F, respectively).

Membrane vesicles (10µg and 30µg of total protein for ABCC2 and ABCC3, respectively) were incubated with different concentrations of CLF in the presence or absence of 4mmol/L ATP. ABCC2 and ABCC3 mediated transport of CLF was concentration-dependent (Fig 4G and H, respectively). The K_m values were $3.3 \pm 2.0 \mu\text{mol/L}$ and $3.7 \pm 1.0 \mu\text{mol/L}$ for ABCC2 and ABCC3, respectively, and $V_{\max}$ values were $436 \pm 215 \text{ pmol CLF/mg protein/min}$ and $188 \pm 55 \text{ pmol CLF/mg protein/min}$, respectively.

Another model substrate for ABCC2 is dinitrophenyl-glutathione (DNP-SG) and CLF should inhibit transport of this compound. Half maximal inhibition of DNP-SG transport with CLF was achieved at approximately $1 \mu\text{mol/L}$ (Fig. 5A). Although no indication was found that CLF is a substrate for ABCB11, it contains the cholyl moiety and therefore, it might be able to inhibit bile salt transport via ABCB11. Indeed, TC transport could be inhibited by CLF in a dose dependent fashion and the concentration at which half maximal transport was observed was approximately $10 \mu\text{mol/L}$ (Fig 5B).

To investigate the role of *Abcc2* and *Abcc3* in plasma clearance of CLF, CLF levels in plasma of wild type, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice after a single injection of CLF in the tail vein were examined. Clearance of CLF was strongly impaired in *Abcc2*^{-/-} mice in comparison with wild type mice but not affected in *Abcc3*^{-/-} mice (Fig 6). To examine the role of *Abcc2* in biliary output of CLF, infusion experiments were performed with wild type and *Abcc2* deficient mice. Biliary excretion of CLF was very much delayed in the *Abcc2*^{-/-} mice (Fig 7A). As a consequence, almost 70% of the CLF dose was excreted into bile in wild type mice within 20 minutes, whereas in the same time span less than 2% was excreted into bile in *Abcc2*^{-/-} mice. At 120 minutes after administration (Fig. 7B), the cumulative biliary excretion of CLF was still significantly higher in wild type mice than in *Abcc2*^{-/-} mice (85% vs 32% of the administered dose) which resulted in a significantly higher hepatic retention of CLF

in *Abcc2*^{-/-} vs wt mice (64% vs 1% of the administered dose) and in higher blood levels in *Abcc2*^{-/-} vs wt mice (1306±749nmol/L vs 83±21nmol/L). These data indicate that, in mice, *Abcc2* is the main transporter responsible for the biliary excretion of CLF. In line with the observations from fig. 7A, it was observed that biliary output of CLF was not affected in *Abcc3*^{-/-} mice in comparison with wild type mice and no differences in plasma and liver CLF contents were found (Fig 7B).

After excretion into bile and delivery into the duodenum, bile salts are taken up in the ileum via the apical sodium dependent bile acid transporter (ASBT) [Wong MH et al, J Biol Chem 1995 Nov 10;270(45):27228-27234]. To investigate whether CLF is taken up in the intestine, equimolar amounts of CLF and [³H]TC were injected into duodenum or ileum of wild type FVB mice. Thereafter, bile was collected and the amounts of [³H]TC and CLF excreted in bile were quantified. At 4.5 hours after ileal injection of both compounds, only 2% of the CLF was recovered in bile whereas this was 68% for [³H]TC. Hence, uptake of CLF in the terminal ileum is minimal. This shows that CLF is not a good substrate for the Asbt.

Conclusions

The results of the investigations into uptake of CLF show that uptake of CLF into hepatocytes is not likely to be mediated by NTCP, since no uptake of CLF into CHO cells expressing human NTCP was found. This was not due to a non-functional protein, since these cells were fully capable of mediating the uptake of the natural occurring bile salt, TC. The ileal counterpart of hepatic NTCP is ASBT and it mediates transport of conjugated and unconjugated bile salts [Geyer J et al, Arch Pharmacol 2006 Mar;372(6):413-431]. Indirect evidence has been obtained to suggest that CLF is not transported via (murine) Asbt, whereas TC injected in the ileal lumen of wild type mice was very efficiently recovered in bile (almost no CLF was found). These data suggest that both NTCP and Asbt, which are homologous sodium-dependent bile salt transporters, are unable to transport CLF.

Previous studies showed partial sodium-dependent uptake of some, but not all, fluorescent bile salts into rat hepatocytes [Maglova LM et al, Hepatology 1995 Aug;22(2):637-647]. In addition, uptake of cholyl-glycyl-fluorescein into CHO cells expressing rat Ntcp, but not wild type cells, has been demonstrated [Boyer JL et al, Am J Physiol 1994 Mar;266(3 Pt 1):G382-G387]. In contrast to CLF, another bile salt conjugate, taurocholy-chlorambucil, was found to be a substrate for NTCP

[Kullak-Ublick GA et al, Gastroenterology 1997 Oct;113(4):1295-1305]. The latter compound is a conjugate at the 3-OH group of the bile salt whereas CLF is conjugated at the side chain. In this context it is interesting that Baringhaus et al determined the pharmacophore of both NTCP and ASBT and found that the 3-OH group is not essential for transport whereas the acidic side chain is [Baringhaus KH et al, J Lipid Res 1999 Dec;40(12):2158-2168]. The above data is completely in line with this model and indicate a species difference of the substrate specificity of NTCP/Ntcp.

10 The less specific bile salt transporter, OATP1B3, turns out to be a more likely candidate for uptake into the hepatocyte, which fits with the broad substrate specificity of this transporter [Hagenbuch B et al, Pflugers Arch 2004 Feb;447(5):653-665].

15 These data demonstrate, surprisingly but conclusively, that ABCC2/Abcc2 is the most prominent transporter responsible for biliary excretion of CLF and not ABCB11. In mice, the large majority of CLF in plasma is excreted into bile via Abcc2. It may be argued that the substrate specificity of human ABCC2 can be different from that in mice. However, in plasma membrane vesicle assays it is demonstrated that
20 transport of CLF via human ABCB11 is insignificant compared to that via human ABCC2. Our present data show decisively that CLF is not transported via ABCB11.

As described above, Mills *et al.* used *Abcc2* deficient, TR⁻ rats and measured CLF in bile after injection of CLF in the jugular vein [J Hepatol 1999 Oct;31(4):678-684].
25 The cumulative amount of CLF in bile was similar in TR⁻ and normal Wistar rats after 30 minutes. However, based on these new data, it seems the cumulative data was misinterpreted (namely initial transport rates could still be different). The new tests, described herein, performed a similar but more extensive study using wild type and *Abcc2*^{-/-} mice. In mice deficient in *Abcc2*, biliary excretion of CLF was strongly
30 impaired and retained in the liver, which suggests that *Abcc2* and not *Abcb11* is the major transporter responsible for biliary excretion of CLF. As there is still residual biliary transport of CLF in *Abcc2*^{-/-} mice, a contribution of *Abcb11*, albeit small, cannot be excluded.

35 Loss of MRP2 function is often well tolerated and compensated by the upregulation of other membrane transporters, particularly MRP3 [Nies et. al Pflugers Arch (2007)]

453:643-659]. The investigations show that not only ABCC2, but also ABCC3 mediates the transport of CLF. Therefore if MRP2 is downregulated in patients, this may be compensated by upregulation of MRP3, and the functional activity of the MRP2 and/or MRP3 pathway of the subject can be investigated using CLF.

5

EXAMPLE 2

A study was carried out in twelve healthy volunteers (six males, six females) aged 21-40 years to compare the plasma pharmacokinetics of CLF after a single 15 second intravenous injection of 2mg CLF, in the presence of medication-induced changes in biliary transporter proteins. The study was carried out under conditions of no pre-treatment, pre-treatment with rifampicin, also known as rifampin (a non-specific inducer and inhibitor of hepatic clearance pathways), and pre-treatment with cyclosporine (cyclosporine is a known inhibitor of MRP2 [Jedlitschky et al, Expert
15 Opin Drug Metab Toxicol 2006 Jun 2(3):351-66]).

CLF was obtained from Norgine, Harefield, United Kingdom. Subjects were studied supine. 2 mg CLF was administered on three occasions by 15 second intravenous injection on the morning of day D01 after an overnight fast and rest with the
20 following modalities:

- Treatment R: no pre- or co-medication
- Treatment T1: ambulatory visits on the evening of Day D-7 until the evening of Day-2 for a single evening dose of 600 mg rifampicin; the last dose of rifampicin was administered on the evening of Day D-1 after admission
- 25 • Treatment T2: single dose of 100 mg cyclosporine on the evening of D-1 and on the morning of D01 one hour before administration of CLF.

Venous blood samples were collected before injection and 2, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240 and 360 minutes after dosing (17 samples). The
30 level of CLF in each sample was determined using HPLC.

The time course of the observed median plasma CLF concentrations are presented in Figure 9. It can be seen that pre-treatment with rifampicin resulted in a slight reduction of the elimination and clearance of CLF. Pre- and co-treatment with
35 cyclosporine caused a distinct inhibition of the elimination and clearance of CLF. This study shows that CLF can be used to determine the OATP1B3 activity and/or

MRP2/3 activity of a subject wherein the subject has been pre-treated with drug substrates that affect OATP1B3 activity and/or MRP2/3 activity.

EXAMPLE 3

5

A study was carried out in twelve healthy volunteers (six males, six females) aged 21-40 years to compare the plasma pharmacokinetics of CLF after a single 15 second intravenous injection of 2mg CLF in the presence of medication-induced changes in biliary transporter proteins. Interaction with ursodeoxycholic acid and cloxacillin was studied in this example.

10

CLF was obtained from Norgine, Harefield, United Kingdom. Subjects were studied supine. 2 mg CLF was administered on three occasions by 15 second intravenous injection on the morning of day D01 after an overnight fast and rest with the following modalities:

15

- Reference R: no pre-treatment
- Treatment T1: pre-treatment and co-treatment with 1 g cloxacillin t.i.d. for three days from the morning of D-3 until the morning of Day D01 0:30 h before administration of CLF
- Treatment T2: three week pre-treatment with daily doses of 500 mg b.i.d ursodeoxycholic acid (in the morning and in the evening each time with food) until the evening of Day D-1

20

Venous blood samples were collected before injection and 2, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240 and 360 minutes after dosing (17 samples). The level of CLF in each sample was determined using HPLC.

25

The time course of the observed median plasma CLF concentrations are presented in Figure 10. It can be seen that pre- and co-treatment with cloxacillin (a known BSEP inhibitor) shows a slight, but statistically significant inhibition of the elimination and clearance of CLF. In contrast, a pre-treatment with ursodeoxycholic-acid had no effect (ursodiol is a known multi-factorial enhancer and facilitator of hepatic biliary clearance).

30

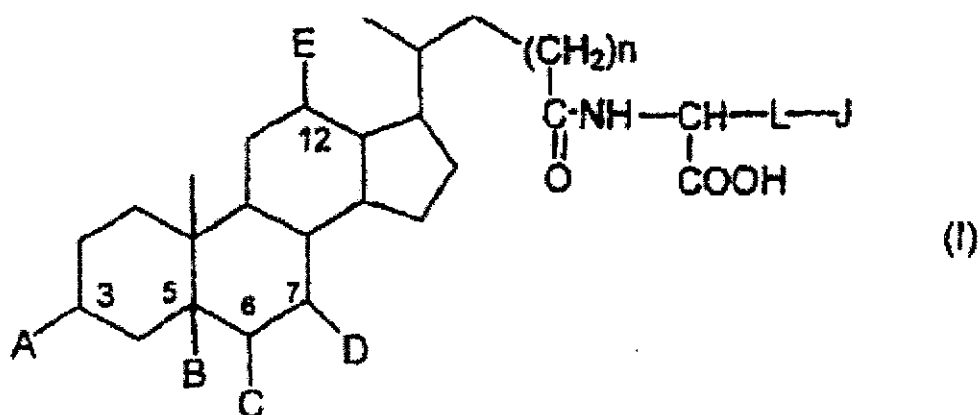
These examples show that CLF can be used to determine medication induced changes in MRP2/3 activity of a subject.

35

Claims:

1. A method of determining the functional activity of the MRP2 and/or MRP3 efflux pathway of a human or animal subject, said method comprising:-

- 5 (i) determining the level of a bile acid derivative of the general formula I,



in the blood of said human or animal subject at a predetermined time interval after introducing an amount of the bile acid derivative into the subject; and

- 10 (ii) using the determination obtained in step (i) to indicate the functional activity of the MRP2 and/or MRP3 efflux pathway of the subject,

wherein A is selected from the group consisting of α -OH and β -OH; B is selected from the group consisting of α -H and β -H; C is selected from the group consisting of -H, α -OH and β -OH; or B and C together form a double bond; D is selected from the group consisting of -H, α -OH and β -OH; E is selected from the group consisting of -H, α -OH and β -OH; L is a linking moiety; J is a coloured or fluorescent moiety; and n is 0 or 1.

15

2. A method according to claim 1 wherein the method comprises a further step of using the determination obtained to arrive at a numerical value, wherein the numerical value indicates the functional activity of the MRP2 and/or MRP3 efflux pathway of the subject.

20

3. A method according to claims 1 or 2 wherein the determination obtained is compared with at least one standard to indicate the functional activity of the MRP2 and/or MRP3 efflux pathway of the subject.

25

4. A method according to any preceding claim wherein the level of bile acid derivative in the blood of said human or animal subject is determined at two or more predetermined time intervals after introducing an amount of said bile acid derivative into the subject.
- 5
5. A method according to claim 4 wherein the method further comprises the step of determining a slope of a log-linear plot of the determinations obtained against time.
- 10
6. A method according to claim 4 wherein the method further comprises the step of determining an area under a plasma efflux curve derived from the determinations obtained.
- 15
7. A method according to any preceding claim wherein the bile acid derivative comprises a marker such as a colour or a fluorescence.
8. A method according to any preceding claim, the method further comprising the step of providing at least one sample of blood which has passed through the liver of the subject and which sample has been collected at a predetermined time interval after administering the bile acid derivative to the subject, wherein the blood sample(s) are processed to obtain blood plasma or blood serum.
- 20
9. A method according to claim 8 wherein the blood sample(s) are processed by centrifugation.
- 25
10. A method according to claims 8 or 9 wherein blood proteins are separated from the blood plasma/blood serum.
11. A method according to any preceding claim wherein the bile acid derivative is administered to the subject intravenously.
- 30
12. A method according to any preceding claim wherein the bile acid derivative comprises (a) a steroid moiety having (i) at least one substituent selected from the group consisting of a 3-hydroxyl substituent, a 7-hydroxyl substituent and a 12-hydroxyl substituent, and (ii) a carboxyl group attached by means of an amide linkage to a side chain of the steroid moiety; and (b) an active moiety which is to be
- 35

targeted to the liver, said active moiety being attached to an α -carbon atom relative to the carboxyl group.

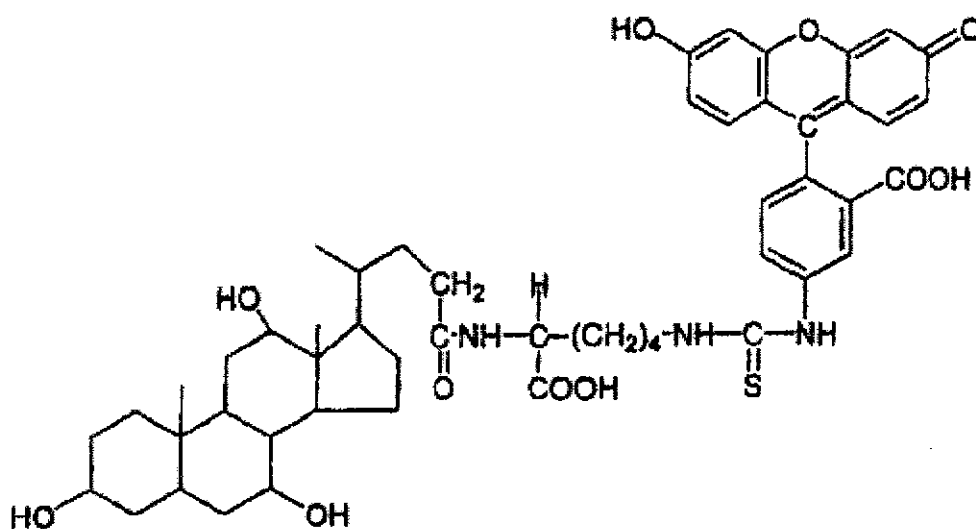
13. A method according to any preceding claim, wherein J is or includes a fluorescein, rhodamine or other fluorescing moiety.

14. A method according to claim 13 wherein the linking moiety is N-terminated at its end attached to active moiety J, and L is selected from the group consisting of:-
 $(\text{CH}_2)_n\text{NH}$, where n is 3 or 4,
 10 $-(\text{CH}_2)_4\text{NH}-(\text{CH}_2)_3\text{NHC}(=\text{NH})\text{NH}-$, and
 $-(\text{CH}_2)_2-\text{CH}(\text{OH})\text{CH}_2\text{NH}-$.

15. A method according to claim 13 wherein the moiety $-\text{NH}-\text{CH}(\text{COOH})-\text{L}-$ in the general formula (I) is derived from a compound selected from the group consisting of S-adenosylhomocysteine, S-adenosylmethiodine, S-aminoimidazole-4-carboxamide, asparagine, cadaverine, cystamine, citrulline, diaminopimelic acid, 2,4-diaminobutyric acid, cysteamine, glutamine, 3-hydroxykynurenine, kynurenine, putrescine and negamycin.

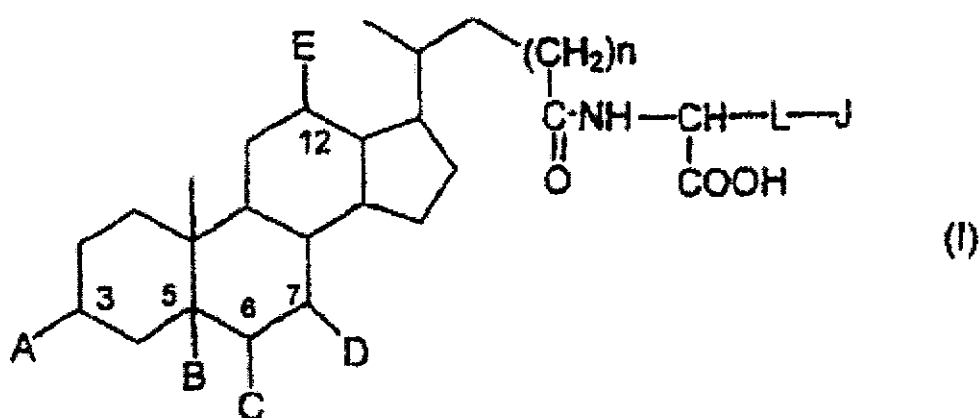
20 16. A method according to claim 12, wherein the steroid moiety of the bile acid derivative is based on an acid selected from the group consisting of cholic acid, chenodeoxycholic acid, deoxycholic acid, hyodeoxycholic acid, hyocholic acid, α -, β - or ω -muricholic acid, nor-bile acids, lithocholic acid, 3 β -hydroxycholenoic acid, ursodeoxycholic acid and allocholic acid (5α -cholan-24-oic-acid).

25 17. A method according to any preceding claim, wherein the bile acid derivative is a cholyl-lysyl-fluorescein (CLF) having the formula:-



18. A method according to claim 17, wherein the bile acid derivative is a salt of
5 cholyl-lysyl-fluorescein.

19. Use of a bile acid derivative of the general formula I for determining the functional activity of the MRP2 and/or MRP3 efflux pathway of a human or animal subject



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wherein A is selected from the group consisting of α -OH and β -OH; B is selected from the group consisting of α -H and β -H; C is selected from the group consisting of -H, α -OH and β -OH; or B and C together form a double bond; D is selected from the
15 group consisting of -H, α -OH and β -OH; E is selected from the group consisting of -

H, α -OH and β -OH; L is a linking moiety; J is a coloured or fluorescent moiety; and n is 0 or 1.

5 20. The use of a determination of MRP2 and/or MRP3 functional activity obtained from the method of any of claims 1 to 18 for determining the liver function of a human or animal subject.

10 21. A method of determining the functional activity of the MRP2 and/or MRP3 efflux pathway of a human or animal subject substantially as herein described.

22. Use of a bile acid derivative for determining the functional activity of the MRP2 and/or MRP3 pathway of a human or animal subject substantially as herein described.

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