# Clinical Investigation on Endogenous Biomarkers to Predict Strong OAT-Mediated Drug–Drug Interactions

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2 **mediated Drug-Drug interactions**

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# Abstract

*Introduction* Endogenous biomarkers are promising tools to assess transporter-mediated drug-drug interactions (DDI) early in humans.

*Methods* We evaluated on a common and validated in vitro system the selectivity of 4-pyridoxic acid (PDA), homovanillic acid (HVA), glycochenodeoxycholate-3-sulfate (GCDCA-S) and taurine towards different renal transporters, including multidrug resistance-associated protein (MRP), and assessed the in vivo biomarker sensitivity towards the strong organic anion transporters (OAT) inhibitor probenecid at 500mg every 6h to reach close to complete OAT inhibition.

*Results* PDA and HVA were substrates of the OAT1/2/3, OAT4 (PDA only) and MRP4; GCDCA-S was more selective, having affinity only towards OAT3 and MRP2. Taurine was not a substrate of any of the investigated transporters under the in vitro conditions tested. Plasma exposure of PDA and HVA significantly increased and the renal clearance of GCDCA-S, PDA and HVA decreased; the magnitude of these changes was comparable to the ones of known clinical OAT probe substrates. PDA and GCDCA-S were the most promising endogenous biomarkers of the OAT pathway activity: PDA plasma exposure was the most sensitive to probenecid inhibition, and, in contrast, GCDCA-S was the most sensitive OAT biomarker based on renal clearance, with higher selectivity towards the OAT3 transporter.

*Conclusion* The current findings illustrate a clear benefit of measuring PDA plasma exposure during Phase 1 studies when a clinical drug candidate is suspected to be an OAT inhibitor based on in vitro data. Subsequently, combined monitoring of PDA and GCDCA-S in both urine and plasma is recommended in order to tease out the involvement of OAT1/3 in the inhibition interaction.

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48    **Key points :**

49    • Selectivity and sensitivity of HVA, PDA, GCDCA-S and taurine were evaluated in vitro with

50       validated and common devices and in vivo with probenecid 500 mg QID allowing close to

51       complete OAT inhibition.

52    • PDA and GCDCA-S were the most sensitive to OAT inhibition based on plasma and urine levels,

53       respectively. PDA has affinity towards multiple renal transporters, whereas GCDCA-S was more

54       selective, transported only by OAT3 and MRP2.

55    • Measurement of PDA in the early Phase 1 studies when only plasma is sampled is recommended

56       for a compound suspected to be an OAT inhibitor. Combined monitoring of PDA and GCDCA-S

57       in urine and plasma is recommended to tease out the involvement of OAT1/3 in the inhibition

58       interaction.

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# 1 Introduction

Inhibition of enzymes and transporters can be a source of drug-drug interactions (DDIs), which can lead to a change in exposure of the active drug and potentially to the appearance of side effects. Therefore, regulatory health agencies and the international transporter consortium (ITC) established guidelines in order to identify and characterize potential DDIs during the development of a new molecular entity (NME) [1-3]. With regards to transporters, a classical approach consists first of performing in vitro tests to identify any potential DDI risk, and subsequently conduct a clinical DDI study towards the transporter for which a potential liability was identified in vitro. Clinical DDI studies usually occur when the development of the NME is already advanced. However, identifying the magnitude of a potential DDI as early as possible is critical during the drug development, to assess and mitigate safety risks.

Predictive tools such as mechanistic static models or physiologically-based pharmacokinetics (PBPK) models can be utilized to estimate the magnitude of the in vivo interaction by using in vitro results [4-8]. However, static models might lead to false negative predictions and despite great progress, predictivity of transporter DDIs with PBPK models remains still challenging. Another approach emerging recently is the use of endogenous biomarkers as an early clinical readout of transporter-mediated DDIs, which could be a cost-efficient and time-saving approach to evaluate DDI early in humans [2, 6, 9]. The benefits of endogenous biomarkers are multiple: first the kinetics of endogenous biomarkers can be evaluated already during the first clinical studies (Phase 1); second, the results of the endogenous biomarker assessment will guide further clinical development and might avoid performing a dedicated DDI study if no change in the kinetics of the endogenous biomarker is observed in presence of the NME. Considering the critical decisions taken upon the results of endogenous biomarker measurements, it remains crucial to characterize their specificity, selectivity and sensitivity to certify the robustness of their use as a readout of transporter DDI [2]. In other words, the change in endogenous biomarker kinetics should ideally reflect the interaction of the inhibitor with the activity of one single transporter, and the extent of change should be similar as the one of clinically used DDI probe drugs [2, 6]. Endogenous biomarkers like coproporphyrins (CPP) are well

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93     documented as a readout of hepatic DDIs mediated by organic anion-transporting polypeptide (OATP) and

94     multidrug resistance-associated protein (MRP) transporters [9-13], whereas additional efforts need to be

95     undertaken for the characterization of endogenous biomarkers of renal transporters such as organic anion

96     transporters (OATs). Recently, Shen et al. identified 4-pyridoxic acid (PDA) and homovanillic acid (HVA)

97     as promising endogenous biomarkers of OAT1 and OAT3, respectively [14, 15]. Tsuruya et al. further

98     identified taurine and glycochenodeoxycholate-3-sulfate (GCDCA-S), as biomarkers of OAT1 and OAT3,

99     respectively [16]. By using internal in vitro systems, both groups characterised the selectivity of these

100    endogenous biomarkers towards different transporters: OAT1/3 [16] and additionally (among others)

101    OAT2/4 and OATP1B1/3 in Shen et al. [15]. The in vitro evaluation was further combined with assessment

102    of the biomarker's sensitivity of plasma and urine levels in Japanese and Indian male populations after

103    different oral single doses of probenecid (500 to 1500mg), the recommended OAT inhibitor in clinical DDI

104    studies [17].

105    In this study, we aim to provide a comprehensive characterization of the selectivity and sensitivity of PDA,

106    HVA, GCDCA-S and taurine towards several renal transporters. For the first time a common in vitro system

107    per transporter was used to characterize selectivity of the four endogenous biomarkers. Specifically, the

108    selectivity was studied towards the basolateral transporters OAT1/2/3/4, the organic cation transporter

109    (OCT)2 and, for the first time, towards MRP2/4, located on the apical membrane of the kidney cells. The

110    clinical DDI sensitivity of these endogenous biomarkers was subsequently evaluated in healthy volunteers

111    towards repeated doses of 500mg probenecid, enabling maximum OAT inhibition. The potential interaction

112    of the probenecid dosing regimen on the endogenous OATP biomarkers CPPI/III in plasma and urine was

113    also assessed, since GCDCA-S is known as a biomarker of OATP activity [18, 19], and to exclude that the

114    changes in GCDCA-S in presence of probenecid are due to OATP inhibition instead of OAT inhibition.

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## 2 Material and Methods

The Material and Methods section is provided in the electronic supplementary material (Online Resource) and includes detailed information about the compounds, the protocol of in vitro experiments, an overview of the design of the clinical study, a description of the bioanalytical methods [20] [Pijpers et al (in preparation)], and the methods used for data analysis. During the clinical study, no concomitant medication, including JNJ compound, that could affect the transporter activity or the synthesis of PDA, HVA, GCDCA-S and taurine was identified (aside from probenecid).

## 3 Results

### 3.1 In vitro renal transporter substrate assessment of PDA, HVA, GDCA-S and taurine

Uptake velocities of the endogenous compounds in transporter overexpressing and control cell lines with final incubation conditions are summarized in Table 1. Transporter reference substrates were included in all experiments, resulting in uptake ratios (overexpressing/control) ranging between 3.3 and 48.0, indicating that the in vitro systems adequately expressed the respective transporter.

As listed in Table 1, the endogenous compounds of interest showed no affinity for OCT2 since uptake ratios were below 1 or the velocities used to calculate the uptake ratio were based on concentrations equal to the lower limit of quantification (LLQ). The uptake velocity of PDA was higher in OAT1, OAT2, OAT3 and OAT4 overexpressing cell lines and MRP4 overexpressing vesicles compared to control cells and vesicles resulting in uptake ratios of >176.6, 20.1, >6.8, 2.5 and >5.2, respectively. PDA concentrations measured in the MDCK-II parental cell line and the HEK293 control vesicles of the MRP4 experiment were below the LLQ and were set to the LLQ in order to compute a velocity of uptake. Therefore, the uptake ratio for OAT3 and MRP4 is likely to be higher because the uptake velocity of PDA in the corresponding control systems is probably overestimated. Moreover, high variability in the velocity of uptake was observed in MRP4 vesicles. No differences in PDA uptake velocity was observed between MRP2 overexpressing vesicles and control vesicles.

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142   A higher uptake velocity of HVA was observed in OAT1, OAT2 and OAT3 overexpressing cells versus

143   control cells, with uptake ratios of 19.8, 19.2 and 2.2, respectively. However, the uptake ratio for OAT3

144   transporter is likely to be higher since a 15 min incubation at a high HVA concentration (50 µM) was

145   required to quantify HVA in the in vitro systems. These conditions are unlikely to represent linear

146   conditions, and therefore a potential underestimation of the uptake ratio is probable. No affinity of HVA

147   with OAT4 was observed. HVA was identified as a MRP4 substrate (uptake ratio = 6.7), but not a MRP2

148   substrate.

149   GCDCA-S was only a substrate for the OAT3 and MRP2 transporters, with uptake ratios of 4.0 and 4.5,

150   respectively. GCDCA-S was not a MRP4 substrate (uptake ratio = 1.4), with GCDCA-S being hardly

151   quantifiable in the control vesicles at the reported conditions of incubation (n=1). No affinity of taurine

152   with the investigated transporters was observed: taurine uptake velocities were comparable between

153   overexpressing and control cell lines.

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155   **3.2    Probenecid inhibition potency towards the studied transporters**

156   Probenecid inhibitor potency was evaluated using well established transporter reference substrates towards

157   transporters for which uptake fold ratio of the endogenous biomarker PDA, HVA and GCDCA-S was at

158   least higher than 2 (Table 1), namely OAT1/2/3 and MRP2/4. The inhibitor potency of probenecid was also

159   evaluated towards the hepatic transporters OATP1B1/3, which are involved in the uptake of CPP-I and

160   CPP-III in hepatic cells. Inhibitor potential of probenecid towards OAT4 was not evaluated in vitro since

161   probenecid is documented as being a weak inhibitor of OAT4, with an in vitro half maximal inhibitory total

162   concentration (IC50) ranging between 56 and 134 µM [15]. Probenecid inhibited OAT1 and OAT3, with

163   an in vitro IC50 of $25.7 \pm 2.8$ µM and $4.67 \pm 0.07$ µM, respectively (Fig 1) and was a weak inhibitor of

164   OATP1B1 (IC50 = $157 \pm 77$ µM). At the highest concentration of probenecid tested (300 µM), only 22%

165   and 11% inhibition of MRP2 and MRP4 activity was observed respectively, and no inhibition of the activity

166   of OAT2 and OATP1B3 was shown.

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### 3.3    Unbound fraction of the biomarkers in plasma

169     Recovery of the incubated concentrations of the four endogenous biomarkers was very high, ranging

170     between 86 and 113%. HVA and taurine were moderately bound to plasma proteins, with $fu_p$ (as fraction)

171     equal to $0.65 \pm 0.13$ and $0.80 \pm 0.01$, respectively. PDA was highly bound to plasma proteins ($fu_p =$

172     $0.081 \pm 0.0004$), as already observed by Shen et al. [14]. GCDCA-S was extremely bound to plasma

173     proteins ($0.0018 \pm 0.0001$), at a value 10-fold lower than the one reported in the literature using

174     ultracentrifugation technique [16]. To confirm our internal result for GCDCA-S, multiple possible variables

175     were assessed (different in vitro systems, non-specific binding, time to reach equilibrium), and all

176     conditions gave the same $fu_p$ value (Online Resource).

177

### 3.4    Clinical assessment of PDA, HVA, GCDCA-S and taurine biomarker sensitivity

179     During 1 week, a 500mg dose of probenecid was administered orally approximately every 6h,

180     corresponding to 4 doses per day (QID), to 6 Caucasian females. The mean plasma concentration-time

181     profile of probenecid is displayed in Fig 2. Probenecid concentrations increased during the first day to reach

182     a mean trough concentration ($C_{trough}$) of 83,767 ng/mL (294 μM) at 24h, corresponding to the time at which

183     the probenecid plasma concentrations reached steady-state. Mean exposure to probenecid 24h after 3rd

184     probenecid doses of 500mg QID probenecid, represented by the area under the plasma concentrations-time

185     curve ($AUC_{0-24h}$), was equal to 5.6 mM.h; the actual plasma exposure is most likely higher, since the $AUC_{0-24h}$

186     of 5.6 mM.h was computed based on $C_{trough}$ probenecid concentrations. It is important to emphasize that

187     the probenecid exposure in this study was at least 1.8-fold higher and ~ 1.4-fold higher than the ones

188 Table 1. Comparison of the uptake velocities of PDA, HVA, GCDCA-S, taurine and transporter reference

189 substrates obtained in uptake transporter-expressing systems and control systems.

| Transporter | In vitro system | Compound (incubation time) | Transporter-overexpressing system (pmol/min/mg.prot) | Control system (pmol/min/mg.prot) | Uptake fold ratio (-) |
|---|---|---|---|---|---|
| OAT1 | CHO cell line | 5 µM PAH (*3 min*) | 38.8 ± 3.4 | 0.8 ± 0.2 | 48.0 |
| | | 10 µM PDA (*1 min*) | 188.0 ± 10.2 | <1.1 * | > 176.6 |
| | | 50 µM HVA (*1 min*) | 617.7 ± 34.3 | 31.2 ± 1.6 | 19.8 |
| | | 10 µM GCDCA-S (*1 min*) | 6.2 ± 3.2 | 17.4 ± 9.7 | 0.4 |
| | | 1 µM taurine (*5 min*) | 3.2 ± 0.3 | 3.7 ± 0.4 | 0.8 |
| OAT2 | HEK293 cell line | 2 µM cGMP (*2 min*) | 54.1 ± 2.4 | 1.5 ± 0.5 | 35.6 |
| | | 10 µM PDA (*5 min*) | 56.2 ± 3.6 | 2.8 ± 0.3 | 20.1 |
| | | 50 µM HVA (*1 min*) | 2091.0 ± 28.8 | 109.1 ± 4.4 | 19.2 |
| | | 10 µM GCDCA-S (*1 min*) | 17.9 ± 3.6 | 19.4 ± 5.3 | 0.9 |
| | | 1 µM taurine (*2 min*) | 8.3 ± 0.3 | 11.1 ± 0.5 | 0.7 |
| OAT3 | MDCK-II cell line | 1 µM E3S (*3 min*) | 35.7 ± 1.1 | 0.9 ± 0.0 | 39.4 |
| | | 10 µM PDA (*1 min*) | 71.0 ± 4.7 | < 11.4 * | >6.8 |
| | | 50 µM HVA (*15 min*) | 7.0 ± 0.6 | 3.2 ± 0.4 | 2.2[a] |
| | | 10 µM GCDCA-S (*1 min*) | 33.7 ± 2.0 | 8.4 ± 2.6 | 4.0 |
| | | 1 µM taurine (*5 min*) | 1.5 ± 0.5 | 1.5 ± 0.1 | 1 |
| OAT4 | HEK293 cell line | 1 µM E3S (*2 min*) | 34.6 ± 0.6 | 0.9 ± 0.1 | 38.9 |
| | | 10 µM PDA (*5 min*) | 6.4 ± 0.5 | 2.5 ± 0.1 | 2.5 |
| | | 50 µM HVA (*5 min*) | 40.7 ± 1.6 | 36.4 ± 1.9 | 1.1 |
| | | 10 µM GCDCA-S (*5 min*) | 3.2 ± 0.3 | 3.7 ± 1.0 | 0.9 |
| | | 15 nM taurine[b] (*5 min*) | 0.03 ± 1.2e-3 | 0.03 ± 1.9e-3 | 1 |
| OCT2 | CHO cell line | 10 µM metformin (*3 min*) | 34.8 ± 0.6 | 1.3 ± 0.2 | 26.6 |
| | | 10 µM PDA (*5 min*) | < 2.0 * | < 1.1 * | NA |
| | | 50 µM HVA (*1 min*) | 15.4 ± 1.3 | 31.2 ± 1.6 | 0.5 |
| | | 10 µM GCDCA-S (*1 min*) | 3.6 ± 0.4 | 17.4 ± 9.7 | 0.2 |
| | | 1 µM taurine (*5 min*) | 1.3 ± 0.1 | 4.0 ± 0.1 | 0.3 |
| MRP2 | HEK293 vesicles | 10 µM CDCF (*5 min*) | 29.3 ± 10.8 | 9.0 ± 6.3 | 3.3 |
| | | 10 µM PDA (*5 min*) | 21.7 ± 0.1 ** | 51.8 ± 29.1 | 0.4 |
| | | 50 µM HVA (*5 min*) | 48.6 ± 29.6 | 128.8 ± 94.5 | 0.4 |
| | | 10 µM GCDCA-S (*5 min*) | 133.2 ± 25.3 | 29.7 ± 7.5 | 4.5 |
| | | 1 µM taurine (*5 min*) | 42.6 ± 4.8 | 37.0 ± 2.6 | 1.2 |
| MRP4 | HEK293 vesicles | 0.2 µM DHEAS (*5 min*) | 29.0 ± 1.9 | 1.2 ± 1.0 | 24.1 |
| | | 10 µM PDA (*5 min*) | 38.0 ± 27.4 ** | < 7.3 * | > 5.2 |
| | | 50 µM HVA (*5 min*) | 200.7 ± 41.4[c] | 29.9± 8.8 | 6.7 |

| | 10 µM GCDCA-S (*5 min*) | 15.9 ± 3.3 | 11.4*** | 1.4 |
| | 1 µM taurine (*5 min*) | 46.0 ± 7.8 | 34.7 ± 4.7 | 1.3 |

PAH = p-aminohippurate, cGMP = cyclic guanosine monophosphate, E3S = esterone -3- sulfate, CDCF = carboxydichlorofluorescein, DHEAS = dehydroepiandrosterone sulfate.

[a]: ratio might be underestimated. HVA was not quantifiable at shorter times of incubation: velocity of uptake might not be measured in linear condition using our quantification method

[b]: data for 1 µM not available

[c] : n = 2. The third measurement was an outlier (value was 10-fold lower than the mean).

NA : not applicable. Concentrations used to compute the velocity were equal to the lower limit of quantification (LLQ) in both the overexpressing and control cell lines

* : concentrations of the three replicates were below the LLQ. Concentrations used to compute the velocity were set to the LLQ for the three replicates.

** n=2, other measured concentrations was below the LLQ

***n=1, other measured concentrations were below the LLQ
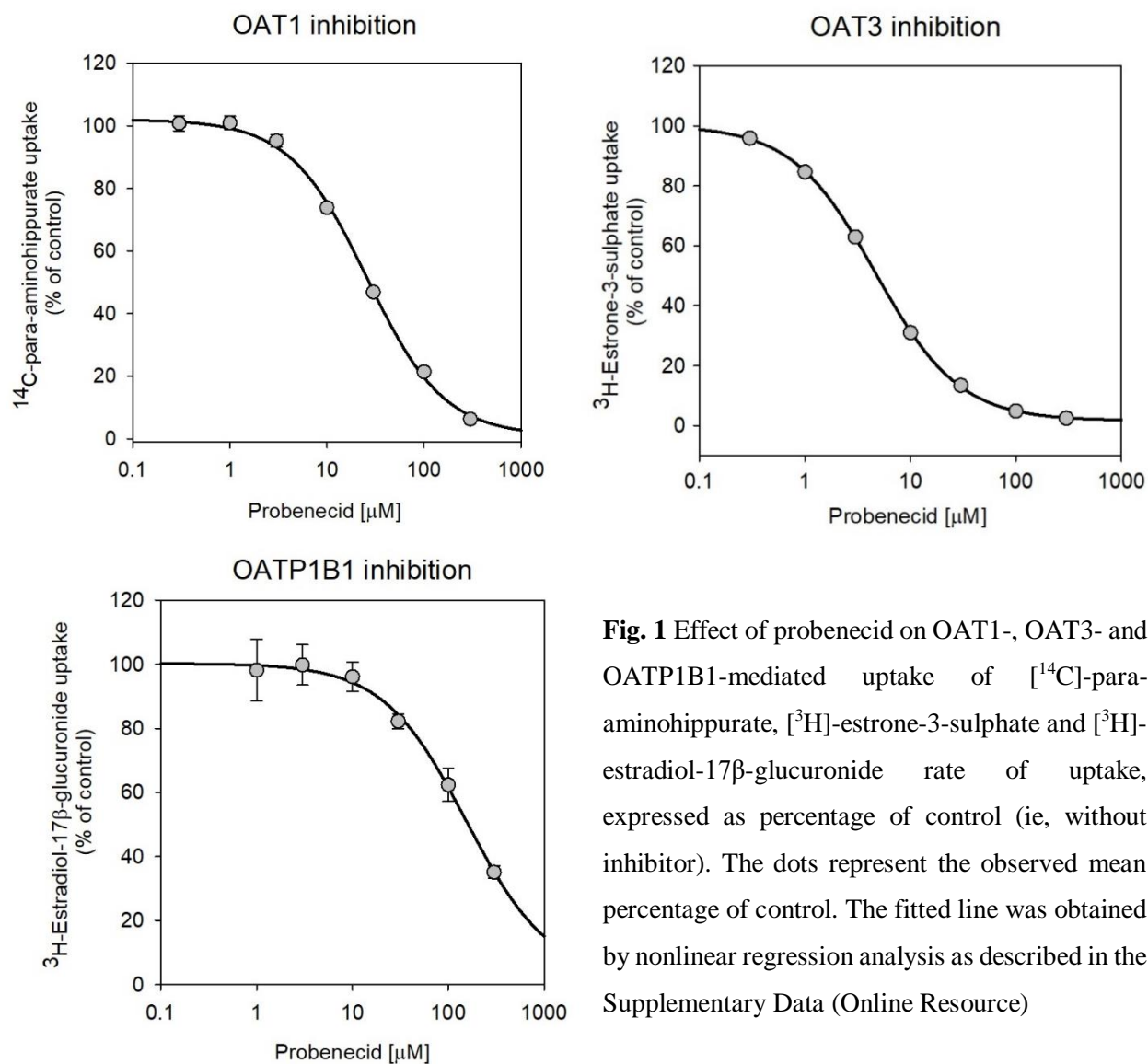


**Fig. 1** Effect of probenecid on OAT1-, OAT3- and OATP1B1-mediated uptake of [$^{14}$C]-para-aminohippurate, [$^{3}$H]-estrone-3-sulphate and [$^{3}$H]-estradiol-17β-glucuronide rate of uptake, expressed as percentage of control (ie, without inhibitor). The dots represent the observed mean percentage of control. The fitted line was obtained by nonlinear regression analysis as described in the Supplementary Data (Online Resource)

214  previously reached for similar evaluation of the effect of probenecid 1000 mg SD on HVA and PDA kinetics

215  (probenecid $AUC_{0-24h}$ = 3.2 mM.h) [14] and probenecid 1500 mg SD on GCDCA-S and taurine kinetics

216  (probenecid $AUC_{0-8h}$ = ~3.9 mM.h) [16], respectively. Therefore, the dosing regimen currently used enabled

217  to maximise the OAT inhibitor potential of probenecid and allow to deconvolute the contribution of OAT

218  mediated uptake in the human pharmacokinetics (PK) of these biomarkers.

219  The effect of the multiple doses of probenecid on PDA, HVA, GCDCA-S and taurine kinetics 24h after the

220  $3^{rd}$ probenecid doses from the 500mg QID probenecid regimen are illustrated in Table 2 and Fig 3.

221  In the absence of probenecid, PDA had a stable baseline, with a mean predose concentration of 3.01 $\pm$

222  0.83 ng/mL (Fig 3 a); predose term corresponds to the blood sampling time before the administration of the

223  JNJ compound and probenecid (Online Resource). In presence of probenecid, all PDA plasma

224  concentrations increased up to 5.6-fold, leading to a significant increase of the $AUC_{0-24h}$ by 3.7-fold

225  (p<0.001). The PDA amount excreted in urine in the 24h time period non-significantly ($Xe_{0-24h}$) decreased

226  by 1.6-fold, and the PDA renal excretion clearance ($CL_R$) significantly decreased by 6.0-fold (p<0.001).

227  Overall, PDA kinetics were sensitive to probenecid (500mg QID), reflected in a change in both plasma and

228  urine matrices.

229  Like PDA, in absence of probenecid, plasma profile of HVA remained stable at 10.1 $\pm$ 3.3 ng/mL (predose)

230  (Fig 3 b). After probenecid administration, each HVA concentration increased by 2.0 to 2.8-fold, triggering

231  a significant increase in $AUC_{0-24h}$ plasma by 2.1-fold (p<0.001). The amount of HVA $Xe_{0-24h}$ non-

232  significantly decreased by 1.4-fold and the $CL_R$ significantly decreased by 3.0-fold (p<0.01). Non-

233  significancy of the change in $Xe_{0-24h}$ compared to the other PK parameters for PDA and HVA can be

234  explained by probenecid having opposite effect with a similar magnitude on plasma $AUC_{0-24h}$ and $CL_R$.

235  HVA kinetics was sensitive to probenecid (500mg QID) on both plasma and urine levels, but to a lesser

236  extent than PDA.

237  Taurine concentration at predose was 6,892 $\pm$ 1,310 ng/mL in absence of probenecid and slightly increased

238  in the presence of probenecid, reaching a maximum at 7h (30% increase), followed by a return to baseline

at 12h (Fig 3 c). The amount of taurine $Xe_{0-24h}$ and the $CL_R$ decreased significantly (p<0.01) on average by 3.2-fold and by 3.4-fold, respectively. Taurine was sensitive to probenecid (500mg QID) on urine level only.
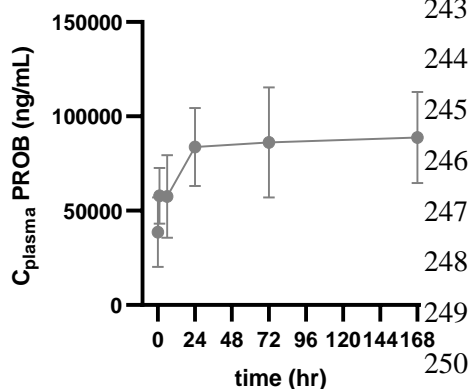


**Fig. 2** Mean and standard deviation of plasma concentration profiles of probenecid (PROB) following 500 mg oral administration of probenecid approximately every 6h (1 week, n=6). Reported concentrations correspond to trough levels (so before the next probenecid administration). At 0h, 2 doses of probenecid 500 mg with 6h in between were already administered. Description of the clinical trial is available in the Supplementary data (Online Resource)

In the absence of probenecid, GCDCA-S showed a bimodal plasma profile with maximal concentration ($C_{max}$) observed at 1h and 12h, and baseline of $29 \pm 10$ ng/mL (predose) (Fig 3 d). In the presence of probenecid, the bimodal plasma profile was still observed but the difference between baseline and $C_{max}$ was more pronounced, with a significant 2.0-fold mean increase in $C_{max}$ (p<0.01). Plasma $AUC_{0-24h}$ significantly increased by 1.9-fold (p<0.01), and the $Xe_{0-24h}$ and the $CL_R$ significantly decreased by 5.1 (p<0.001) and 9.5-fold (p<0.001), respectively. Overall, the GCDCA-S kinetics were sensitive to probenecid (500mg QID), with urine parameters providing the most sensitive readouts of all four biomarkers investigated.

For PDA, HVA, taurine and GCDCA-S, inter-individual variability in the plasma PK parameters $C_{max}$ and $AUC_{0-24h}$ was moderate with and without probenecid (CV < 30%), in contrast to high variability seen in urine parameters $Xe_{0-24h}$ and $CL_R$ (CV >90% for PDA and taurine), with reduced CVs observed in presence of probenecid (CV = 61% for PDA and 65% for taurine). Difference in the inter-individual variability between plasma and urine PK parameters can be explained by the higher contribution of the renal elimination pathway compared to non-renal elimination pathways in the total elimination of these biomarkers. Interindividual differences in renal transporter activity will have a more pronounced effect on

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266 renal elimination than on plasma concentrations. This is further substantiated by a significant decrease in

267 the variability of renal clearance and amounts excreted in urine in presence of probenecid.

268 Fig 4 illustrates the correlation between the plasma concentrations of endogenous biomarkers and the total

269 probenecid plasma concentrations, over the time range 0 to 168h (full time range of the treatment). PDA

270 and HVA plasma concentrations correlated with increased total probenecid plasma concentrations, starting

271 approximately at probenecid plasma concentrations of 50,000 ng/mL ($r^2 = 0.44$ for PDA and 0.51 for HVA)

272 However, no such trend was observed for taurine and GCDCA-S.

273

274 **3.5    Clinical Assessment of CPP-I and CPP-III sensitivity to probenecid**

275 In the absence of probenecid, CPP-I and CPP-III profiles were stable, at a concentration (predose) of 0.375

276 $\pm$ 0.071 ng/mL and 0.034 $\pm$ 0.011 ng/mL, respectively (Fig 3 e and f). The inter-individual variability for

277 both CPP-I and CPP-III in the plasma and urine kinetics parameters were low (CV = 15 - 25%) and high

278 (CV = 50 – 63%), respectively. In the presence of probenecid, CPP-I and CPP-III plasma $AUC_{0-24h}$ showed

279 a small but significant (p<0.01) increase by 1.4 and 1.6-fold, respectively. This increase is however

280 negligible compared to the plasma increase observed with a potent OATP inhibitor (eg 7-fold for CPP-I

281 and CPP-III after single rifampicin dose, compared to placebo group [10]). The $CL_R$ of CPP-I and CPP-III

282 decreased by 1.2-fold: these changes were not significant because of the high inter-individual variability in

283 the urine related parameters. No clear correlation was observed between CPPs plasma concentration and

284 probenecid plasma concentrations (Fig 4 e and f). Overall, CPP-I and CPP-III showed limited sensitivity to

285 probenecid (500mg QID) in plasma, with no effect on their renal elimination in line with the in vitro results

286 that probenecid is not a potent inhibitor of OATP/MRP and that CPPs are not a substrate of OATs.

287

288    Table 2. Comparison of the pharmacokinetic plasma and urine parameters $AUC_{0-24h}$, $C_{max}$, $Xe_{0-24h}$ and $CL_r$

289    for PDA, HVA, taurine, GCDCA-S, CPP-I and CPP-III before and 24h after $3^{rd}$ dose of 500 mg QID

290    probenecid in 6 healthy volunteers.

| Analyte/Parameter | | Treatment A (no probenecid) | | | Treatment B (with probenecid) | | | GMR | |
|---|---|---|---|---|---|---|---|---|---|
| | | mean | | SD | mean | | SD | | |
| **PDA** | | | | | | | | | |
| $AUC_{0-24h}$ | (hr*ng/mL) | 71.8 | ± | 21.3 | 271 | ± | 93 | *** | 3.7 ↑ |
| $C_{max}$ | (ng/mL) | 3.6 | ± | 0.8 | 13.7 | ± | 4.1 | *** | 3.7 ↑ |
| $Xe_{0-24h}$ | (µg/kg BW) | 22.5 | ± | 21.0 | 13.1 | ± | 8.0 | | 1.6 ↓ |
| $CL_R$ | (mL/min/kg BW) | 4.7 | ± | 2.9 | 0.77 | ± | 0.36 | *** | 6.0 ↓ |
| **HVA** | | | | | | | | | |
| $AUC_{0-24h}$ | (hr*ng/mL) | 247 | ± | 61 | 536 | ± | 165 | *** | 2.1 ↑ |
| $C_{max}$ | (ng/mL) | 13.4 | ± | 3.8 | 34.9 | ± | 10.7 | *** | 2.6 ↑ |
| $Xe_{0-24h}$ | (µg/kg BW) | 85.4 | ± | 45.8 | 58 | ± | 16.4 | | 1.4 ↓ |
| $CL_R$ | (mL/min/kg BW) | 5.8 | ± | 2.8 | 1.9 | ± | 0.7 | ** | 3.0 ↓ |
| **taurine** | | | | | | | | | |
| $AUC_{0-24h}$ | (hr*ng/mL) | 166,480 | ± | 22,582 | 180,848 | ± | 23,810 | | 1.1 ↑ |
| $C_{max}$ | (ng/mL) | 8,970 | ± | 1,668 | 10,005 | ± | 2,118 | | 1.1 ↑ |
| $Xe_{0-24h}$ | (µg/kg BW) | 1,268 | ± | 1,165 | 374 | ± | 243 | ** | 3.2 ↓ |
| $CL_R$ | (mL/min/kg BW) | 0.127 | ± | 0.123 | 0.034 | ± | 0.022 | ** | 3.4 ↓ |
| **GCDCA-S** | | | | | | | | | |
| $AUC_{0-24h}$ | (hr*ng/mL) | 858 | ± | 259 | 1,574 | ± | 395 | ** | 1.9 ↑ |
| $C_{max}$ | (ng/mL) | 53.5 | ± | 16.7 | 104.4 | ± | 20.1 | ** | 2.0 ↑ |
| $Xe_{0-24h}$ | (µg/kg BW) | 4.5 | ± | 2.4 | 0.8 | ± | 0.3 | *** | 5.1 ↓ |
| $CL_R$ | (mL/min/kg BW) | 0.088 | ± | 0.048 | 0.009 | ± | 0.005 | *** | 9.5 ↓ |
| **CPP-I** | | | | | | | | | |
| $AUC_{0-24h}$ | (hr*ng/mL) | 9.07 | ± | 1.53 | 12.77 | ± | 2.57 | ** | 1.4 ↑ |
| $C_{max}$ | (ng/mL) | 0.456 | ± | 0.068 | 0.625 | ± | 0.115 | ** | 1.4 ↑ |
| $Xe_{0-24h}$ | (µg/kg BW) | 0.296 | ± | 0.151 | 0.350 | ± | 0.122 | | 1.2 ↑ |
| $CL_R$ | (mL/min/kg BW) | 0.566 | ± | 0.344 | 0.446 | ± | 0.125 | | 1.2 ↓ |
| **CPP-III** | | | | | | | | | |
| $AUC_{0-24h}$ | (hr*ng/mL) | 0.833 | ± | 0.205 | 1.4 | ± | 0.4 | ** | 1.6 ↑ |
| $C_{max}$ | (ng/mL) | 0.054 | ± | 0.01 | 0.072 | ± | 0.015 | * | 1.3 ↑ |
| $Xe_{0-24h}$ | (µg/kg BW) | 0.821 | ± | 0.516 | 0.990 | ± | 0.374 | | 1.3 ↑ |
| $CL_R$ | (mL/min/kg BW) | 16.6 | ± | 9.8 | 12.6 | ± | 5.8 | | 1.2 ↓ |

291 Table 2 footnote: Description of the clinical trial is available in the Supplementary data (Online Resource). A two-
292 tailed paired Student's *t*-test was applied to evaluate the effect of probenecid. $AUC_{0-24h}$ : area under the plasma
293 concentrations-time curve, $C_{max}$ : maximal plasma concentration, $Xe_{0-24h}$ : amount eliminated in urine, $CL_r$ : renal
294 clearance and GMR : geometric mean ratio. GMR is back-transformed from log scale of the ratio of pharmacokinetic
295 parameter with probenecid over without probenecid (when the ratio is increasing) * p<0.05, ** p< 0.01, ***p< 0.001,
296 when the parameter was compared to without probenecid

## 4    Discussion

298 Validated endogenous biomarkers of transporter activity could become an essential tool in early clinical

299 assessment of transporter-mediated DDI. In this study we performed a comprehensive characterization of

300 four endogenous biomarkers on validated in vitro systems. The selectivity was studied towards the renal

301 OATs (OAT1/2/3/4), OCT2 and for the first time the efflux transporters MRP2/4. Finally, the in vivo

302 translatability was assessed by testing the sensitivity of the four biomarkers both on plasma and urine levels

303 to probenecid 500mg QID in 6 subjects. This probenecid dosing regimen enabled to reach more potent

304 OAT inhibition than in previous studies, in which a single dose of probenecid was studied, resulting in, at

305 least, a 1.4- to 1.8-fold lower plasma probenecid exposure compared with the current study. Power

306 calculation to inform the design of prospective studies with PDA as endogenous biomarker confirmed the

307 suitability of small number of subjects when investigating strong OAT1/3 inhibitors (such as probenecid)

308 under current multiple dosage regimen design, whereas the requirements for evaluation of moderate-weak

309 inhibitors differ [21].

310 Based on the in vitro assays, PDA and HVA were found to be substrates of the three OATs (OAT1/2/3)

311 renal uptake transporters. Additional putative contributors to the transport of these substrates included:

312 OAT4 (PDA only) and MRP4 (PDA and HVA). Only the uptake transporter OAT3 and the efflux

313 transporter MRP2 were involved in the GCDCA-S kinetics, therefore making GCDCA-S a more selective

314 biomarker of renal OAT3/MRP2 transporters compared to PDA and HVA. In this study, taurine was not a

315 substrate of any of the transporters tested, including in cells stably expressing OAT1 and OAT3, which is

316 in line with previous observations in similar in vitro system [16]. Tsuruya et al. (2016) identified taurine as

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317 an OAT1 substrate on stable cell lines overexpressing OAT1, in which OAT1 level seem higher than in

318 transient cell lines. Our results on the involvement of OATs in the transport of the four endogenous

319 biomarkers are overall confirmatory of previous reports [14, 16]. The uptake ratios for GCDCA-S towards

320 OAT4 (0.9 in our study, 1.6 in Tsuruya et al. [16]) were both below the threshold of 2 established by the

321 US FDA, which therefore does not classify GCDCA-S an OAT4 substrate. However, we reported PDA as

322 an OAT2 and OAT4 substrate with uptake ratios above 2, in contrast to the findings of Shen et al. (2017),

323 which could be explained by a difference in the transporter expression levels in the cells line system used

324 between the studies.

325 Comparison of in vitro transporter inhibition potencies of probenecid with measured plasma concentration

326 of probenecid after 500mg QID enables identification of the likely mediators of observed clinical

327 interaction with endogenous biomarkers. Considering a probenecid $fu_p$ of 0.10 for a mean $C_{trough}$ probenecid

328 plasma concentration at 24h of 294 µM [22], free plasma probenecid concentration circulated at, at least,

329 29 µM. Actual free plasma probenecid concentration is most likely higher since considerations are on $C_{trough}$

330 and probenecid $fu_p$ increases in a nonlinear manner with probenecid plasma concentration increase,

331 reaching a maximal $fu_p = 0.26$ at 1051 µM [22]. In our study, circulating probenecid concentration exceeded

332 therefore the in vitro IC50 of OAT1 and OAT3. Thus potent in vivo inhibition of OAT1 and OAT3

333 transporters was expected. Conversely, in vivo OATP1B1 inhibition was expected to be minimal, while

334 MRP2, MRP4, OAT2/4 and OATP1B3 were not inhibited at all. The lack of clinical relevance of the in

335 vitro inhibition results towards OATP1B3, OATP1B1 and MRP2 with the current probenecid regimen was

336 confirmed by the CPP results: plasma and urine CPP levels, biomarker of the OATP/MRP pathway,

337 remained relatively stable after probenecid 500mg QID. Additionally, these results confirmed the lack of

338 contribution of OAT in the renal clearance of CPPs [10]. The effect of probenecid on GCDCA-S with the

339 current probenecid regiment is hence not confounded by OATP inhibition. Due to endogenous nature of

340 these biomarkers, changes in plasma exposure in presence of probenecid may also reflect the effect on their

341 synthesis, and not only their elimination. There is no evidence that probenecid affects the formation of

342 either PDA or HVA, but consequences of this scenario were evaluated in our companion paper [21].

343  The OAT substrates, recommended by the health authorities for clinical DDI evaluation, show an increase

344  of their plasma exposure and a decrease of their urine excretion in presence of a potent OAT inhibitor. For

345  example, in the case of adefovir (OAT1 substrate) and benzylpenicillin (OAT3 substrate), a 1.9- and 3.2-

346  fold increase in plasma AUC and a 2- and 4.5-fold decrease in the $CL_R$ were observed, respectively, in

347  presence of probenecid 1500mg single dose [14, 23]. Based on the US FDA guidelines, an OAT-mediated

348  DDI  is considered significant if the plasma AUC of the substrate increases by 1.5-fold in presence of the

349  inhibitor [24]. These criteria are helpful for the identification of a relevant OAT endogenous biomarkers.

350  In our clinical study, 3 (PDA, HVA and GCDCA-S) out of 4 endogenous biomarkers expressed their

351  sensitivity to probenecid by a significant change in both plasma and urine related parameters; taurine

352  presented a significant decrease in urine related parameters only. Increase in $AUC_{0-24h}$ plasma or decrease

353  of $CL_R$ of the endogenous biomarkers were within the same magnitude as the ones observed for the OAT

354  reference substrates.

355  The following rank order in sensitivity of endogenous biomarkers to probenecid was identified: PDA >

356  HVA > GCDCA-S based on plasma $AUC_{0-24h}$ increases, and GDCDCA-S > PDA > taurine > HVA based

357  on $CL_R$ decreases. However, the interpretation of these changes should be considered together with the low

358  and high inter-individual variability observed for plasma and urine parameters, respectively. PDA plasma

359  data provided then the most sensitive readout of OAT1/2/3 inhibition, followed by HVA. As PDA is a

360  vitamin B6 metabolite: the intake of this vitamin should be controlled during the clinical studies to avoid

361  any misinterpretation of the cause of variation in the PDA baseline level. Monitoring GCDCA-S was

362  beneficial because of its higher selectivity, as being transported only by OAT3 (among OATs), in contrast

363  to PDA and HVA which are transporter substrates of OAT1/2/3. However, GCDCA-S, a bile acid sulfate,

364  has the drawback of a variable baseline over time, with a diurnal change in its plasma profile (Fig 3 d).

365  Additionally, in contrast to PDA, GCDCA-S has affinity for certain hepatic transporters.
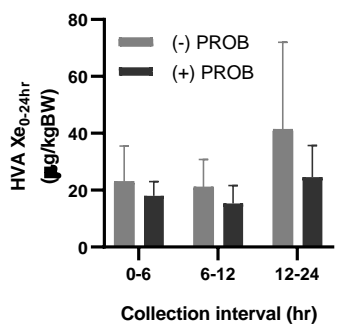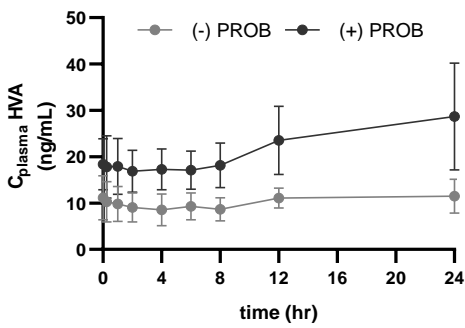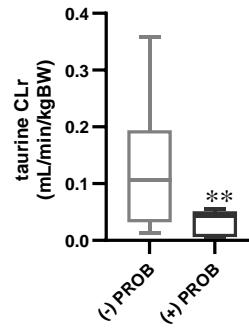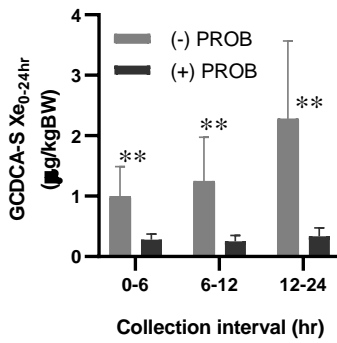
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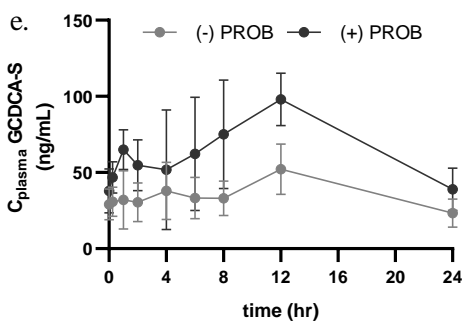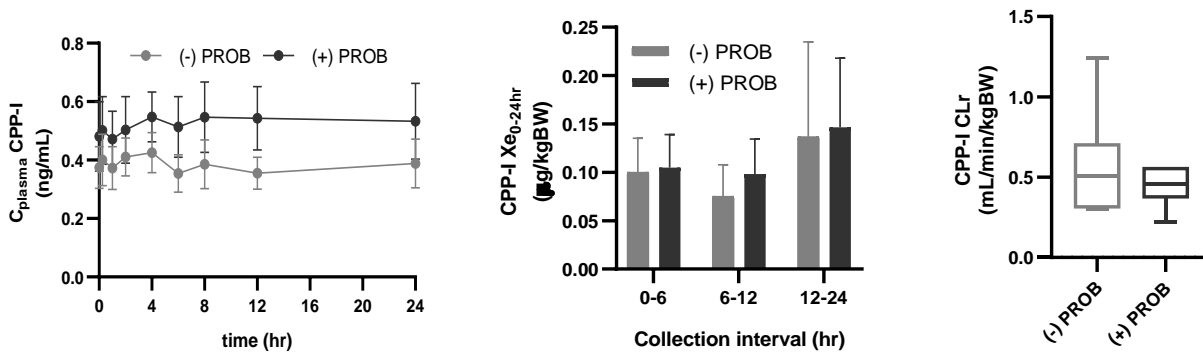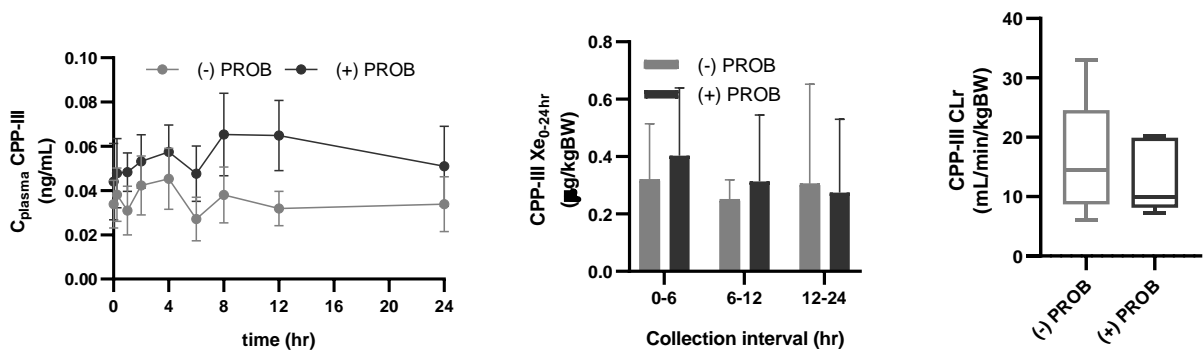

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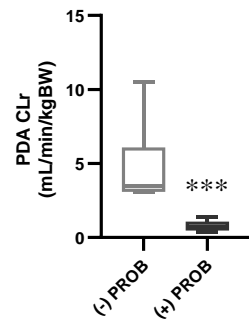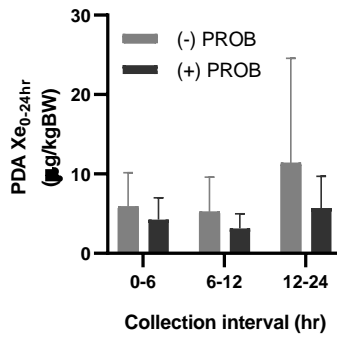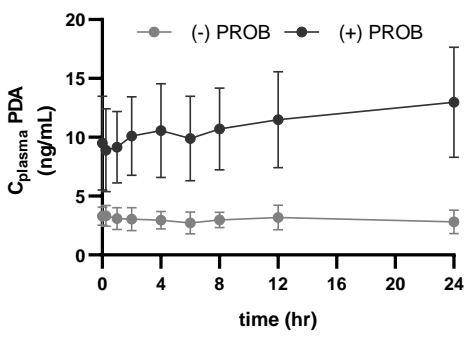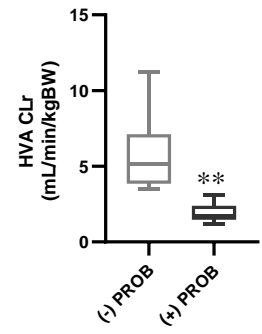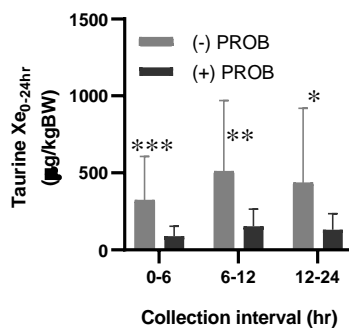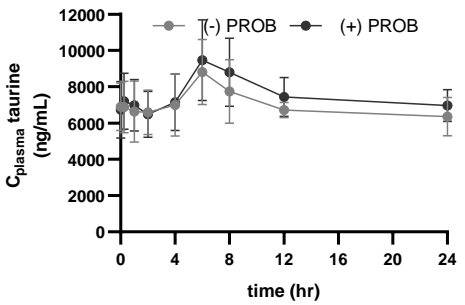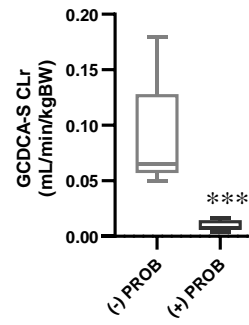
**Fig. 3** Mean and standard deviation of plasma concentration-time profiles, amount eliminated in urine (Xe0-24h) and renal clearance (CLr) of PDA (a), HVA (b), taurine (c), GCDCA-S (d), CPP-I (e) and CPP-III (f) alone and 24h after 3rd dose of 500 mg QID probenecid (PROB), in 6 healthy volunteers. Description of the clinical trial is available in the Supplementary data (Online Resource). A two-tailed paired Student's t-test was performed, * p<0.05, ** p< 0.01, ***p< 0.001, when the parameter (Xe0-24h or Clr) was compared to without probenecid

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385  Our study and Shen et al. [15] identified MRP2 and the hepatic transporter OATP1B1, respectively, as

386  being involved in the kinetics of GCDCA-S; GCDCA-S was also proposed as a clinical biomarker of OATP

387  inhibition [25, 26]. However, with CPP levels in plasma and urine remaining relatively stable in presence

388  of probenecid in the current clinical study, we were able to confirm that GCDCA-S change can be fully

389  attributed to OAT3 inhibition, excluding contribution of OATP/MRP in this context. In general, this lack

390  of selectivity towards transporters, as observed for GCDCA-S, can be a confounding factor for a biomarker,

391  but in case of a suspected strong OATP inhibition it can be addressed by measuring GCDCA-S urine

392  concentrations and calculate renal clearance to facilitate interpretation of renal DDI. In view of NME

393  development process, measurement of PDA in Phase 1 studies when only plasma is sampled is envisaged

394  to provide a valuable initial evaluation of DDI risk (unless very strong OAT3 inhibition and lack of OAT1

395  inhibition was observed in vitro). When urine and plasma are sampled, monitoring of both PDA and

396  GCDCA-S would enable further deconvolution of the impact of inhibitors on OAT1/OAT2 and

397  OAT3/MRP2.

398  The analysis of urine data in our clinical study also gave insights in the mechanisms and the transporters

399  involved in the renal excretion of endogenous biomarkers investigated. The $CL_R$ of PDA, HVA and

400  GCDCA-S was 14.8, 2.3 and 12.5-fold higher than $fu_p \times$ measured glomerular filtration rate (GFR) in

401  absence of probenecid, and 2.4-fold higher (PDA), 30% lower (HVA) and 30 % higher (GCDCA-S), after

402  probenecid administration. These results indicated a substantial active renal secretion of PDA and GCDCA-

403  S and to a lesser extent for HVA, driven probably by the uptake and efflux transporters identified in our in

404  vitro studies with some active secretion remaining for PDA after probenecid administration (potentially due

405  to remaining OAT1 activity, or OAT2 and MRP4 involvement). Other efflux transporters on the basolateral

406  side not investigated in our study (e.g., MRP1/3) may contribute. Finally, for taurine, the $CL_R$ was 23-fold

407  and 224-fold lower than the corrected GFR without and with probenecid, respectively, highlighting that the

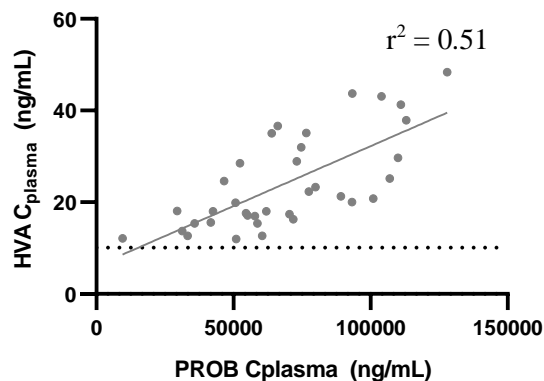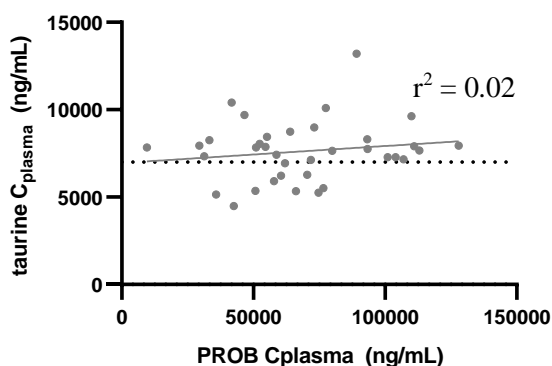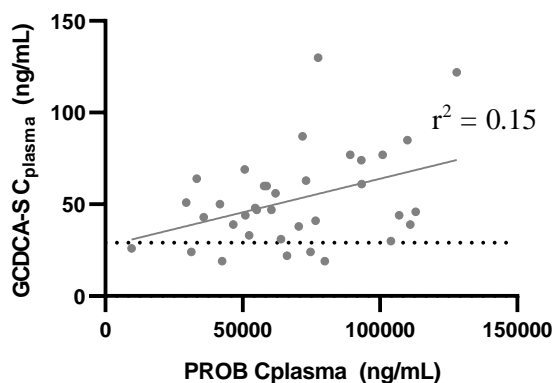408  renal elimination was likely driven by reabsorption and secretion.

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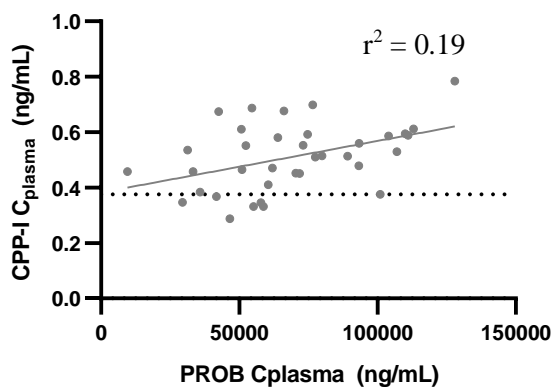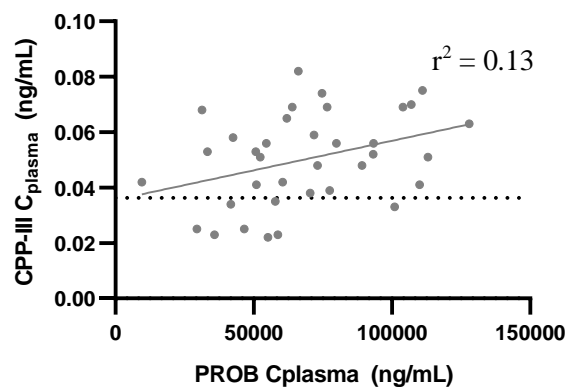
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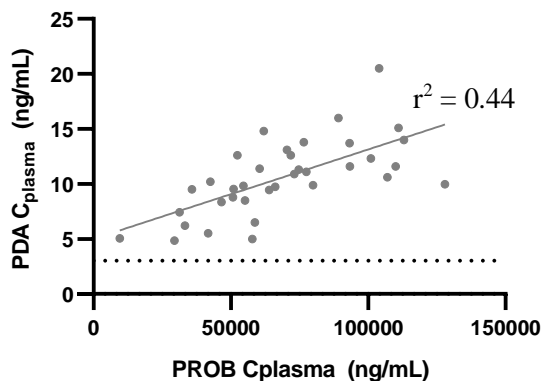
**Fig. 4** Correlation between the plasma concentrations of PDA (a), HVA (b), taurine (c), GCDCA-S (d), CPP-I (e) and CPP-III (f) and probenecid (PROB) plasma concentrations. The respective plasma concentrations were determined from 36 plasma samples. The dotted line represents the mean concentration of endogenous biomarker at predose (before administration of probenecid)

418    The reabsorption of taurine can potentially be explained by the involvement of uptake transporters on the

419    apical membrane not investigated in this study [16], e.g., the sodium-dependent transporter (Taut) [27].

420    With the current design of the clinical study, probenecid $AUC_{0-24h}$ plasma was at least 1.4-to 1.8-fold higher

421    than the ones reported in previous studies [14, 16] enabling to reach close to complete OAT1/3 inhibition,

422    especially for OAT3, which has a 5.5-fold lower IC50 of probenecid compared to OAT1. Therefore, the

423    deconvolution of the OAT-mediated pathway of the biomarkers versus passive filtration was correctly

424    assessed in our study which is crucial to define the dynamic range for clinical OAT inhibition. Urine

425    parameters of the endogenous biomarkers were the most sensitive to this higher probenecid plasma

426    exposure. Indeed, OAT1/3-related secretion (ie, $CL_r$ in presence of probenecid) of PDA, HVA and

427    GCDCA-S represented 83%, 67% and 90% of their total $CL_r$ (ie, $CL_r$ in absence of probenecid),

428    respectively, whereas percentages based on previous results were lower, at 71%, 63% and 89%, respectively

429    [14, 16]. Higher percentages observed for HVA and PDA was probably because OAT1 was more inhibited

430    in our study compared to previous studies [14, 16]. For GCDCA-S, a more specific OAT3 substrate, the

431    minimal difference observed in OAT mediated secretion between this study and previous studies can

432    probably be attributed to the lower IC50 of probenecid for OAT3 vs OAT1.

## 5   Conclusions

434    Our results enabled identification of PDA, HVA, GCDCA-S as promising endogenous biomarkers of OAT

435    renal transporters, by characterizing their selectivity and clinical sensitivity with regard to potent OAT

436    inhibition. The findings highlight the PDA plasma data as the most informative early OAT biomarker.

437    Combined monitoring of PDA and GCDCA-S in both plasma and urine allows evaluation of contributions

438    of multiple transporters, specifically OAT1 and OAT3. The sensitivity of these biomarkers needs to be

439    further consolidated by evaluating the impact of OAT inhibitors with different levels of potency (moderate,

440    mild) on their kinetics. Finally, modelling of the reported clinical data (with and without probenecid) will

441    allow the quantification of the different elimination pathways involved, including the $CL_R$, to support

442    prospective design of OAT interaction studies.

## Declarations

451  **Conflicts of interest** Marie-Emilie Willemin, Ils Pijpers, Lieve Dillen, Sophie Jonkers, Kathleen

452  Steemans, An Tuytelaars, Frank Jacobs, Mario Monshouwer and Jan Snoeys are full-time employees of

453  Janssen Pharmaceutical Companies of Johnson & Johnson.

454  **Ethics approval**  All procedures performed in studies involving human participants were in accordance

455  with the ethical standards of the institutional and/or national research committee and with the 1964

456  Helsinki Declaration and its later amendments or comparable ethical standards.

457  **Consent to participate** Informed consent was obtained from all individual participants included in the

458  study.

459  **Consent for publication** The authors affirm that human research participants provided informed consent

460  for publication

461  **Availability of data and material** The datasets generated during and/or analysed during the current

462  study are available from the corresponding author on reasonable request

463  **Code availability** NOT APPLICABLE

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**Authors contributions**

ME.W., T.VDM., I.P., F.J. and A.G. wrote manuscript. ME.W., T.VDM., A.K., F.J., M.M., D.S., A.R.,

A.G. and J.S. designed research. T.VDM., I.P., S.J., K.S. and A.T. performed research. ME.W., T.VDM.,

I.P., L.D., A.K., S.J., K.S., A.T., F.J. and J.S. analysed data

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