Pharmacokinetic Evaluation of a Drug Transporter Cocktail Consisting of Digoxin, Furosemide, Metformin, and Rosuvastatin

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This article reports the clinical investigation of a probe drug cocktail containing substrates of key drug transporters. Single oral doses of 0.25 mg digoxin (P-gp), 5 mg furosemide (OAT1 and OAT3), 500 mg metformin (OCT2, MATE1, and MATE2-K), and 10 mg rosuvastatin (OATP1B1, OATP1B3, and BCRP) were administered separately or as a cocktail in a randomized six-period crossover trial in 24 healthy male volunteers. As a cocktail, relative bioavailabilities of digoxin and metformin and furosemide $AUC_{0\text{-}tz}$ were similar to separate dosing. However, when administered as a cocktail the C_{max} of furosemide was 19.1% lower and the C_{max} and $AUC_{0\text{-}tz}$ of rosuvastatin were 38.6% and 43.4% higher, respectively. In addition, the effects of increased doses of metformin or furosemide on the cocktail were investigated in 11 and 12 subjects, respectively. The cocktail explored in this trial has the potential to be used for the *in vivo* screening of transporter-mediated drug–drug interactions. © 2016 American Society for Clinical Pharmacology and Therapeutics

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ The "cocktail study" is a valuable approach for the combined investigation of several drug–drug interactions (DDI) in a single clinical trial, and drug cocktails are frequently and successfully used for investigation of CYP-mediated DDI. So far, no drug cocktail consisting of probe substrates for the relevant drug transporters has been validated.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ To establish an *in vivo* drug cocktail consisting of four probe substrates for key drug transporters as recommended by US Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines to examine in a very cost-effective way the potential for a development compound to cause transporter-mediated DDI.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

☑ This is the first report of a clinical trial investigating mutual pharmacokinetic interactions in a drug cocktail that is designed to specifically assess key drug transporters P-gp, OAT1, OAT3, OCT2, MATE1, MATE2-K, OATP1B1, OATP1B3, and BCRP.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

☑ This transporter cocktail, once optimized and fully validated, will be a valuable and efficient tool for investigation of transporter-based DDI in drug development.

Drug transporters are membrane-bound proteins that play an important role in drug absorption, distribution, and excretion.[1] Inhibition of transporters by concomitantly administered drugs may cause clinically relevant drug–drug interactions (DDI).[2–4] Regulatory authorities recommend DDI studies that address the *in vitro* effect of new investigational medicines on P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), organic anion transporting polypeptide 1B1 (OATP1B1), OATP1B3, organic cation transporter 2 (OCT2), organic anion transporter 1 (OAT1), and OAT3.[5,6] Based on guideline-defined cutoff values, if *in vitro* data do not warrant exclusion of an *in vivo* DDI study, clinical trials are typically recommended to examine the potential for an investigational drug to alter the pharmacokinetic profiles of suitable probe drugs for relevant transporters.[5,6] In addition to the seven transporters mentioned previously, the inhibitory effect of new compounds on emerging transporters of potential clinical relevance such as multidrug and toxin extrusion protein 1 (MATE1) and MATE2-K, should be considered.[5–7]

In vitro inhibition cutoff values are generally somewhat conservative, with the intention of ensuring patient safety. The number of drug transporters recognized as clinically relevant from a DDI perspective is continuously expanding, which will most likely lead to an increase in the number of clinical trials during drug development to determine the potential for transporter-mediated DDI. A valuable approach to reduce the number of DDI trials in drug development is the "cocktail study," in which

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a mixture of well-characterized probe drugs is administered together with a new investigational medicine in a single clinical trial to investigate several different DDI mechanisms. This approach is well established for cytochrome P450 (CYP)-mediated DDI and is endorsed by regulatory authorities.[5,6,8,9]

So far, a cocktail consisting of probe drugs for transporters involved in clinically relevant DDI has not been established. Recently, four drugs were proposed as probe substrates for key drug transporters based on *in vitro* investigations.[10] The drugs evaluated were the cardiac glycoside digoxin (P-gp), the loop diuretic furosemide (OAT1 and OAT3), the antidiabetic metformin (OCT2, MATE1, and MATE2-K), and the HMG-CoA reductase inhibitor rosuvastatin (OATP1B1, OATP1B3, and BCRP). Metabolism of these drugs in humans is minor or negligible, allowing for investigation of transporter-mediated DDI without the potential for enzyme inhibition to confound the results. All four drugs have been characterized *in vitro* as substrates of the respective transporters, and interactions with inhibitors of these transporters have been well documented in clinical studies.[1–3,7,10,11] Based on *in vitro* data, mutual interactions involving the four studied drugs via inhibition of their respective transporters were considered unlikely when administered at low doses, as used in this trial.[10]

The principal objective of this clinical trial was to explore the conclusions of the *in vitro* studies described by Ebner *et al.*[10] by studying the proposed four-component transporter DDI cocktail in healthy human subjects. In this trial, using relatively low doses (cocktail T1), the relative bioavailability of each component after administration in the cocktail compared to administration of the respective component as a single entity was determined. In addition, mutual pharmacokinetic interactions were also investigated in two subgroups of volunteers, consisting of a 2-fold increase in the dose of metformin (T2) or a 4-fold increase in the dose of furosemide (T3).

Although the likelihood of mutual interactions between the selected probe drugs was projected to be low based on *in vitro* results,[10] previous reports indicated that *in vivo* interactions could occur with metformin or furosemide as perpetrators, in particular if plasma concentrations of the perpetrators were increased.[12–14] Such an increase could theoretically occur when the cocktail is administered together with a potent inhibitor of transporters, and therefore the T2 and T3 arms were included to investigate this potential effect.

RESULTS

Subjects

Twenty-four healthy white male subjects were randomized, 12 subjects in each trial part, and treated. The median age was 37.5 (range 23–49) years and the mean body mass index 25.6 kg/m^2 (standard deviation [SD] 2.1). Twenty-two subjects completed the planned observation time according to protocol. Two subjects in trial part 1 withdrew consent for further participation, one during period 1, another after period 2. A further subject in trial part 1 did not participate in period 3 (treatment: rosuvastatin alone) at the discretion of the investigator due to a nondrug-related adverse event (nasopharyngitis), but continued as planned with period 4. All 24 subjects (100%) were included in the randomized set and the treated set, and 23 subjects (95.8%) were included in the pharmacokinetic set (PKS). One subject from part 1 was excluded from the PKS because of too few pharmacokinetic samplings for the primary and secondary pharmacokinetic endpoints.

Pharmacokinetics

Digoxin. Geometric mean plasma concentration–time profiles of digoxin when given alone (reference) or as a component of test cocktails T1, T2, and T3 are shown in **Figure 1** and **Supporting Figure S1** and the pharmacokinetic parameters in **Table 1**. Maximum plasma concentrations occurred at a median t_{max} of 1.0 hours after all treatments, and urinary excretion parameters $fe_{0\text{-}36}$ and $CL_{R,0\text{-}36}$ were comparable between the different treatments (**Table S1**). Graphical comparisons of individual and geometric mean $AUC_{0\text{-}tz}$ and C_{max} values are given in **Figure S2**.

The relative bioavailability of digoxin in test cocktail T1 compared to dosing alone was close to 100% for $AUC_{0\text{-}tz}$ and C_{max} (**Table 1**), and the 90% confidence intervals (CIs) were within the standard bioequivalence (BE) acceptance range of 80 to 125%. Relative bioavailability of digoxin in cocktail T2 compared to digoxin alone was also close to 100% for both $AUC_{0\text{-}tz}$ and C_{max}. The 90% CI for $AUC_{0\text{-}tz}$ were within the standard BE range, but the upper 90% CI for C_{max} was slightly above the upper BE limit. Bioavailability of digoxin as part of cocktail T3 compared to dosing alone was decreased by 17.0% with respect to $AUC_{0\text{-}tz}$ and 11.4% with respect to C_{max} (**Table 1**). Bioavailability of digoxin as part of cocktail T3 compared to dosing as part of T1 was decreased by 22.6% with respect to $AUC_{0\text{-}tz}$ and 12.2% with respect to C_{max}. The secondary endpoint $AUC_{0\text{-}\infty}$ was not used for relative bioavailability analysis because it could not be determined with sufficient precision.

Furosemide. Geometric mean plasma concentration–time profiles of furosemide when given alone or as a component of test cocktails T1 and T2 are shown in **Figures 1** and **S1**, and the pharmacokinetic parameters in **Table 2**. Plasma concentration maxima occurred at a median t_{max} of 40 minutes to 1 hour after all treatments, and the urinary excretion parameters $fe_{0\text{-}36}$ and $CL_{R,0\text{-}36}$ were comparable between the treatments (**Table S1**). Graphical comparisons of individual and geometric mean $AUC_{0\text{-}tz}$ and C_{max} values are given in **Figure S3**.

Relative bioavailability of furosemide in cocktails T1 and T2 compared to dosing alone was close to 100% for both $AUC_{0\text{-}tz}$ and $AUC_{0\text{-}\infty}$, and the 90% CIs were within the BE acceptance range (**Table 2**). However, geometric mean C_{max} of furosemide in cocktails T1 and T2 was 19.1% and 21.4% lower, respectively, than that of furosemide alone, and the lower 90% CIs were below the lower BE limit. $AUC_{0\text{-}tz}$ and C_{max} of furosemide were 4.0 and 3.1 times higher, respectively, in cocktail T3 (20 mg furosemide dose) compared to furosemide alone (5 mg dose) (**Table S2**).

Metformin. Geometric mean plasma concentration–time profiles of metformin when given alone (reference) or as a component of test cocktails T1 and T3 are shown in **Figures 1** and **S1**, and the pharmacokinetic parameters in **Table 3**. Plasma concentration maxima occurred at a median t_{max} of 3.0 hours after all

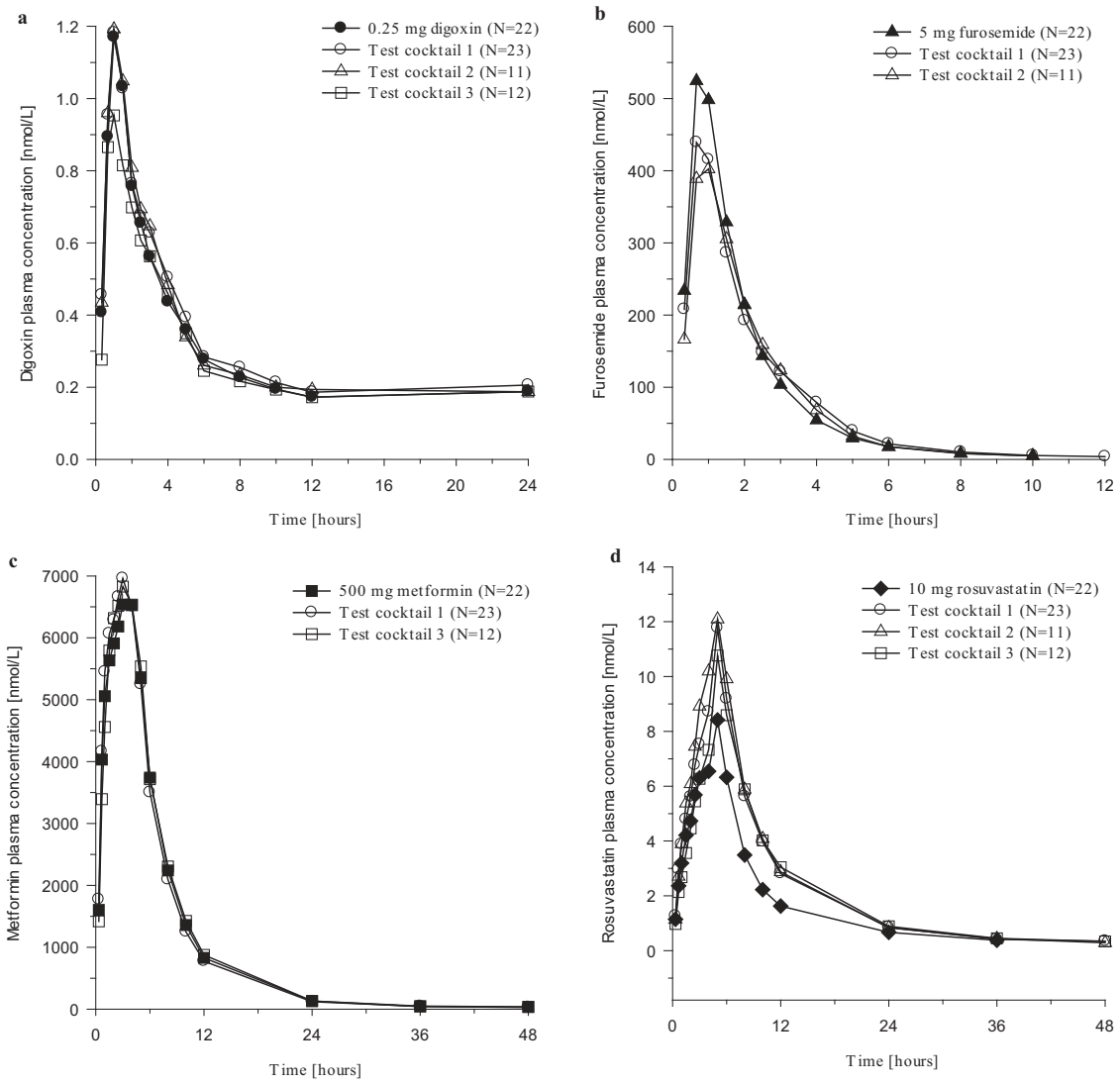

Figure 1 Geometric mean plasma concentration–time profiles of digoxin (**a**), furosemide (**b**), metformin (**c**), and rosuvastatin (**d**). The four compounds were either given alone as monotreatment or all together as test cocktail T1 at doses of 0.25 mg digoxin, 5 mg furosemide, 500 mg metformin hydrochloride, or 10 mg rosuvastatin. In test cocktail T2, a doubled metformin dose was given (1,000 mg), and in test cocktail T3, a 4-fold furosemide dose was given (20 mg) together with the other compounds. Scaling is linear.

treatments, and metformin $fe_{0\text{-}36}$ and $CL_{R,0\text{-}24}$ were decreased by 10–18% in cocktails T1 and T3 compared to metformin alone (**Table S1**). Graphical comparisons of individual and geometric mean $AUC_{0\text{-}tz}$ and C_{max} values are given in **Figure S4**.

Relative bioavailability of metformin in cocktails T1 and T3 compared to dosing alone was close to 100% for $AUC_{0\text{-}tz}$, C_{max}, and $AUC_{0\text{-}\infty}$ (**Table 3**). The 90% CIs were within the BE acceptance range with the exception of the upper 90% CI for C_{max} in T3, which was slightly above the 125% BE acceptance limit. $AUC_{0\text{-}tz}$ and C_{max} of metformin were 1.7- and 1.8-fold higher, respectively, when metformin was given as a 1,000 mg dose as part of cocktail T2, compared with 500 mg metformin given alone (**Table S2**).

Rosuvastatin. Geometric mean plasma concentration–time profiles of rosuvastatin given alone (reference) or in test cocktails T1, T2, or T3 are shown in **Figures 1** and **S1**, and the pharmacokinetic parameters in **Table 4**. Maximum plasma concentrations of rosuvastatin were observed after a median t_{max} of 5.0 hours after all treatments. Rosuvastatin $fe_{0\text{-}36}$ was higher in cocktails T1, T2, and T3 compared to rosuvastatin alone; for example, it increased from 5.45% when dosed alone to 7.93% in cocktail T1. However, rosuvastatin $CL_{R,0\text{-}36}$ in the different treatment periods was comparable (**Table S1**). Graphical comparisons of individual and geometric mean $AUC_{0\text{-}tz}$ and C_{max} values are given in **Figure S5**. Rosuvastatin geometric mean ratios of $AUC_{0\text{-}tz}$, C_{max}, and $AUC_{0\text{-}\infty}$ in the comparison of rosuvastatin in the cocktails to rosuvastatin alone increased by 43.4%, 38.6%, and 27.8%, respectively, in cocktail T1, by 50.9%, 37.9%, and 37.4% in cocktail T2, and by 45.6%, 40.1%, and 23.3% in cocktail T3. The geometric mean ratios of all the above-mentioned pharmacokinetic parameters were themselves

Table 1 Geometric means and relative bioavailability for the primary pharmacokinetic endpoints of digoxin given as a single administered drug (reference) or in test cocktails 1, 2, and 3

	Test cocktail		Reference		Ratio T/R	90% CI	gCV[a]
Endpoint	*N*	Adj. gMean	*N*	Adj. gMean	[%]	[%]	[%]
	Digoxin of Test Cocktail 1 (T1)		Digoxin alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	22	8.78	22	8.18	107.32	(95.41; 120.72)	22.6
C_{max} [nmol/L]	22	1.31	22	1.30	100.51	(89.61; 112.73)	22.1
	Digoxin of Test Cocktail 2 (T2)		Digoxin alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	11	8.00	22	8.00	99.90	(81.11; 123.06)	25.7
C_{max} [nmol/L]	11	1.32	22	1.30	102.03	(82.54; 126.14)	30.6
	Digoxin of Test Cocktail 3 (T3)		Digoxin alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	12	6.80	22	8.19	82.96	(73.77; 93.31)	15.8
C_{max} [nmol/L]	12	1.15	22	1.30	88.56	(77.17; 101.65)	18.7

[a]Intraindividual.

Table 2 Geometric means and relative bioavailability for the primary and secondary pharmacokinetic endpoints of furosemide given as a single administered drug (reference) or in test cocktails 1 and 2

	Test cocktail		Reference		Ratio T/R	90% CI	gCV[a]
Endpoint	*N*	Adj. gMean	*N*	Adj. gMean	[%]	[%]	[%]
	Furosemide of Test Cocktail 1 (T1)		Furosemide alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	22	1018.10	22	1057.87	96.24	(88.61; 104.53)	15.8
C_{max} [nmol/L]	22	476.23	22	588.87	80.87	(71.51; 91.47)	23.7
$AUC_{0\text{-}\infty}$ [nmol·h/L]	22	1039.36	22	1078.85	96.34	(88.77; 104.56)	15.7
	Furosemide of Test Cocktail 2 (T2)		Furosemide alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	11	1034.17	22	1047.13	98.76	(86.16; 113.21)	15.9
C_{max} [nmol/L]	11	459.13	22	583.93	78.63	(67.51; 91.58)	18.4
$AUC_{0\text{-}\infty}$ [nmol·h/L]	11	1048.00	22	1068.58	98.07	(86.09; 111.73)	15.3

[a]Intraindividual.

Table 3 Geometric means and relative bioavailability for the primary and secondary pharmacokinetic endpoints of metformin given as a single administered drug (reference) or in test cocktails 1 and 3

	Test cocktail		Reference		Ratio T/R	90% CI	gCV[a]
Endpoint	*N*	Adj. gMean	*N*	Adj. gMean	[%]	[%]	[%]
	Metformin of Test Cocktail 1 (T1)		Metformin alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	22	49778.86	22	50056.90	99.44	(93.40; 105.88)	11.9
C_{max} [nmol/L]	22	7575.10	22	7083.05	106.95	(97.65; 117.12)	17.2
$AUC_{0\text{-}\infty}$ [nmol·h/L]	22	50380.82	22	50573.02	99.62	(93.66; 105.95)	11.6
	Metformin of Test Cocktail 3 (T3)		Metformin alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	12	51659.75	22	49958.76	103.40	(90.19; 118.56)	19.1
C_{max} [nmol/L]	12	7332.03	22	7067.60	103.74	(84.67; 127.11)	29.9
$AUC_{0\text{-}\infty}$ [nmol·h/L]	12	52189.46	22	50470.30	103.41	(90.31; 118.40)	19.0

[a]Intraindividual.

Table 4 Geometric means and relative bioavailability for the primary and secondary pharmacokinetic endpoints of rosuvastatin given as a single administered drug (reference) or in test cocktails 1, 2, and 3

	Test cocktail		Reference		Ratio T/R	90% CI	gCV[a]
Endpoint	*N*	Adj. gMean	*N*	Adj. gMean	[%]	[%]	[%]
	Rosuvastatin of Test Cocktail 1 (T1)		Rosuvastatin alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	22	103.29	22	72.03	143.39	(128.13; 160.46)	21.0
C_{max} [nmol/L]	22	11.61	22	8.38	138.57	(122.55; 156.69)	23.0
$AUC_{0\text{-}\infty}$ [nmol·h/L]	22	110.80	18	86.68	127.83	(114.59; 142.59)	17.5
	Rosuvastatin of Test Cocktail 2 (T2)		Rosuvastatin alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	11	108.43	22	71.87	150.87	(116.95; 194.62)	27.8
C_{max} [nmol/L]	11	11.57	22	8.39	137.93	(109.31; 174.05)	25.1
$AUC_{0\text{-}\infty}$ [nmol·h/L]	11	125.59	18	91.38	137.43	(113.89; 165.85)	16.6
	Rosuvastatin of Test Cocktail 3 (T3)		Rosuvastatin alone (R)				
$AUC_{0\text{-}tz}$ [nmol·h/L]	12	106.40	22	73.07	145.61	(124.27; 170.61)	20.4
C_{max} [nmol/L]	12	11.98	22	8.55	140.05	(122.88; 159.63)	16.7
$AUC_{0\text{-}\infty}$ [nmol·h/L]	11	114.25	18	92.70	123.25	(94.27; 161.15)	24.3

[a]Intraindividual.

outside the BE limits, except for $AUC_{0\text{-}\infty}$ for cocktail T3, and the 90% CIs were all outside the BE acceptance range (**Table 4**).

Safety

The four probe drugs were safe and well tolerated when given alone or all together as a cocktail. Details on the safety and tolerability are provided in **Table S3** and the **Supplementary Safety** data.

DISCUSSION

This clinical investigation in healthy subjects represents further development of a four-component transporter DDI cocktail that was initially proposed from an exhaustive *in vitro* evaluation.[10] The selection of these specific four probe drugs and their doses, i.e., digoxin (0.25 mg), furosemide (5 mg), metformin (500 mg), and rosuvastatin (10 mg), was also based on their sensitivity to coadministered inhibitors of the respective transporters in previous clinical trials, expected general clinical safety, and broad commercial availability. It was considered important that the probe drugs are not substantially metabolized, as this would complicate interpretation of DDI with coadministered inhibitors of both drug transporters and metabolizing enzymes.

The relative bioavailabilities of the substrates in cocktail T1 compared to when each drug was administered alone were unchanged for metformin and digoxin. Furosemide C_{max} showed a slight (19%) decrease in cocktail T1, which is consistent with a previous report of a 31% and 12% decrease in furosemide C_{max} and AUC, respectively, following concomitant administration of metformin.[12] The slight decrease in furosemide C_{max} observed could therefore be caused by coadministration with metformin. Interestingly, the C_{max} of furosemide decreased in both cocktails T1 and T2, whereas $AUC_{0\text{-}tz}$ and $AUC_{0\text{-}\infty}$ did not relevantly change, suggesting that the underlying mechanism is rather a reduction in the rate of intestinal furosemide absorption as opposed to a change in the extent of bioavailability. This is corroborated by results of cocktail T3, in which, after a 4-fold dose (20 mg) of furosemide, $AUC_{0\text{-}tz}$ showed a 4-fold increase as compared to the reference dose (5 mg), whereas furosemide C_{max} increased less than dose-proportionally (see **Table S2**). Moreover, urinary PK data indicate an extrarenal mechanism of interaction, as no relevant change of furosemide CL_R was observed in cocktails T1 and T3 as compared to monotreatment (**see Table S1**). This is of particular importance given that furosemide is intended as probe drug substrate for renal transporters OAT1 and OAT3. Therefore, the sensitivity of furosemide as *in vivo* indicator of renal OAT modulators should not be influenced by the slight decrease of furosemide C_{max} observed in the cocktail.

For rosuvastatin, the plasma exposure increased by 39% and 43% for C_{max} and $AUC_{0\text{-}tz}$, respectively, in cocktail T1 compared to dosing alone (**Table 4**). This result was unexpected, and the underlying mechanism responsible for the increase in rosuvastatin exposure is presently unclear and may be based on a still unknown molecular mechanism. The increase in the fraction of rosuvastatin excreted in urine without any change in CL_R (**Table S1**) could indicate that an increase in bioavailability, rather than a change in systemic clearance, was responsible for the observed increase in AUC and C_{max} when administered as part of the cocktails. Based on the potential for transporter-mediated DDIs determined *in vitro*, the risk of mutual interactions between the four probe drugs at low single oral doses was considered to be remote.[10] Additionally, the potential for mutual interaction of digoxin, furosemide, and metformin with rosuvastatin based on either MRP2 (expressed in gut and liver), OATP2B1 (expressed in gut and liver), or NTCP (expressed in

liver) is regarded as remote (*in vitro* results: data not shown). This is important, because rosuvastatin is reported to be a substrate of MRP2[15,16] and NTCP,[17,18] whereas the contribution of OATP2B1 is somewhat controversial.[15,19,20]

In cocktail T1 there are 12 potential pairwise interactions, three of which affect rosuvastatin. Clinical DDI studies involving coadministration of metformin and rosuvastatin[13] and digoxin and rosuvastatin[21] have been reported, but coadministration of furosemide and rosuvastatin has not yet been investigated. Lee *et al*.[13] reported a randomized crossover trial in 36 healthy male volunteers, in which metformin (750 mg q.d.) did not alter AUC_{τ} of rosuvastatin 10 mg q.d., but caused a slight increase of rosuvastatin geometric mean $C_{max,ss}$ by 23%. In another trial, rosuvastatin (40 mg q.d.) had no relevant effect on digoxin pharmacokinetics[21] but the effect of digoxin on rosuvastatin was not investigated. Based on the results reported by Lee *et al*.,[13] it is possible that metformin is the perpetrator of the rosuvastatin exposure increase, although causative involvement of digoxin or furosemide cannot be fully excluded.

The magnitude of the rosuvastatin plasma exposure increase must be placed into context. Rosuvastatin, which was selected specifically because of its sensitivity as a probe drug, exhibits marked changes of plasma concentrations on coadministration with inhibitors of OATP and BCRP.[1,11] The observed increase in rosuvastatin exposure in the cocktails is small compared to the effect seen with rifampin, a potent inhibitor of OATP1B1 and OATP1B3 and an inhibitor of BCRP.[22] The concomitant administration of 5 mg rosuvastatin and 600 mg rifampin to eight healthy volunteers resulted in an increase of rosuvastatin plasma C_{max} and $AUC_{0\text{-}24}$ by 9.9-fold and 5.2-fold, respectively.[22]

It would be preferable if no interactions occurred in the cocktail.[5,6] In order to optimize the cocktail in such a way that the interaction is avoided, the dose of the perpetrator could be decreased. In such a case, it would be necessary to both identify the perpetrator and the extent of perpetrator dose reduction necessary to avoid the interaction. Alternatively, rosuvastatin could be replaced by another probe for BCRP and hepatocellular OATPs that shows no interaction with the other cocktail compounds. However, such an alternative probe substrate would first have to be identified. Another possibility could be to investigate in clinical trials whether the sensitivity of rosuvastatin for detection and quantification of transporter-mediated DDI is impaired by the observed interaction in cocktail T1.

Previous reports[12–14] indicated that increased plasma concentrations of metformin or furosemide could potentially cause interactions with the other cocktail compounds. Therefore, two further cocktail compositions were investigated. Cocktail T2 ($n = 11$) contained a doubled metformin dose (1,000 mg) to simulate an increase in metformin exposure if the cocktail were to be given with a potent OCT/MATE inhibitor.[23] Cocktail T3 ($n = 12$) contained a 4-fold increased furosemide dose (20 mg) to simulate a corresponding increase in furosemide plasma concentrations in the presence of a potent OAT inhibitor.[24]

Cocktail T2 demonstrated no further interactions compared to T1, suggesting that cocktail T1 pharmacokinetics would not be relevantly affected in the presence of OCT/MATE inhibitors. Cocktail T3 revealed a potential minor interaction with digoxin (decreases in $AUC_{0\text{-}tz}$ by 17% and C_{max} by 11% comparing T3 vs. R, and decreases of $AUC_{0\text{-}tz}$ by 22.6% and C_{max} by 12.2% comparing T3 vs. T1), without additionally affecting the pharmacokinetics of metformin or rosuvastatin (**Tables 1–4).** This suggests that higher plasma concentrations of furosemide, resulting, for example, from concomitant administration of an OAT1/OAT3 inhibitor, could slightly decrease digoxin plasma concentrations.

It is difficult to derive a mechanistic basis for the decreases of digoxin AUC and C_{max} in cocktail T3 as a result of the higher furosemide dose. Several previous studies examined the effect of furosemide on digoxin pharmacokinetics, but the results were not consistent. Both increases[25] and decreases[26] of digoxin renal excretion were reported after administration of relatively high doses of furosemide. Other groups reported the absence of relevant effects of furosemide on digoxin pharmacokinetics.[27–30] In the current cocktail trial, digoxin CL_R was not increased in cocktail T3, suggesting an extrarenal site of interaction. A possible explanation would be a decrease in the extent of bioavailability, which may be due to a decrease in intestinal digoxin absorption. However, the mechanistic basis for a decrease in digoxin absorption cannot be explained by available *in vitro* and clinical data, and as such, a definitive explanation for this effect remains to be elucidated.

This trial was not powered to demonstrate bioequivalence for all four compounds, but rather to explore the feasibility of a practically manageable number of 24 subjects (with 22 completing the trial) to test transporter DDI interactions of new test substances. For this exploratory trial, no multiplicity adjustment was performed, either for power or for the overall significance level. This should be taken into account when considering the confidence interval data and planning future trials.

One of the prerequisites for the transporter probe substrates was their clinical safety.[10] In the current trial, the four probe drugs administered alone or together as a cocktail were safe and well-tolerated.

In current CYP enzyme cocktails, e.g., the Sanofi-Aventis cocktail,[8] drugs are combined that exhibit metabolic pathways that are nearly exclusively mediated by specific drug-metabolizing enzymes. In contrast, it is more challenging to identify clinically safe, nonmetabolized drugs that are selective substrates for only one specific transporter and where plasma exposures are sufficiently sensitive to modulation of solely this specific transporter.

Three of the four drugs of the transporter cocktail proposed by Ebner *et al*. are intended to function as *in vivo* substrates for more than one relevant transporter.[10] In addition, all four drugs are reportedly *in vitro* substrates for additional drug transporters, although the *in vivo* relevance of these experimental data is thought to be minimal.[10] However, the cocktail investigated in the current trial would be highly valuable for drug development. If transporter-based interactions cannot be excluded for an investigational compound based on *in vitro* results, the cocktail may be used as a screening tool in an initial interaction study in which the effect of the investigational drug on key drug transporters is assessed. Results from such a transporter cocktail DDI study, e.g.,

a change of relative bioavailability of one or more probe drugs, could then trigger suitably designed and more focused follow-up studies. On the other hand, absence of interaction in such a trial could rule out potential DDIs mediated by key drug transporters.

In conclusion, this is the first report of a clinical trial investigating mutual pharmacokinetic interactions in a drug cocktail that is designed to specifically assess key drug transporters. The probe drugs were digoxin (P-gp), furosemide (OAT1 and OAT3), metformin (OCT2, MATE1, and MATE2-K), and rosuvastatin (OATP1B1, OATP1B3, and BCRP). When all four drugs were given together (cocktail T1), geometric mean values for rosuvastatin C_{max} and $AUC_{0\text{-}tz}$ were 38.6% and 43.4% higher than after treatment with rosuvastatin alone. It would be desirable if the cocktail could be improved in such way that the interaction with rosuvastatin is avoided, e.g., by dose reduction of the perpetrator. Alternatively, a suitably designed trial could investigate whether the sensitivity of rosuvastatin for detection and quantification of transporter-based DDI is affected in the cocktail. Unaffected sensitivity of rosuvastatin as a probe would be a strong argument for usability of the cocktail. The small decrease of furosemide C_{max} in cocktail T1 should not affect the sensitivity of furosemide as a probe for DDI on the level of renal OATs. For digoxin, based on the results of our trial, it cannot be excluded that increased plasma exposures of furosemide, e.g., due to interaction with a new investigational drug, could indirectly affect digoxin plasma concentrations.

The transporter cocktail, once optimized and fully validated, will be a valuable and efficient tool for investigation of transporter-based DDI in drug development.

METHODS

Human subject protection

The clinical trial protocol was approved by the Ethics Commission of the State Chamber of Physicians of Baden-Württemberg, Stuttgart, Germany, and the Federal Institute for Drugs and Medicinal Products (BfArM), Bonn, Germany. The trial was conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonisation guidelines for Good Clinical Practice. The EudraCT number was 2014-001940-40, and the trial was registered at ClinicalTrials.gov (identifier: NCT02231931).

Subjects

Twenty-four healthy male subjects aged 18–50 years and with a body mass index (BMI) of 18.5–29.9 kg/m^2 were eligible to participate in the trial. Females were not included to avoid any potential interference of hormonal cycle or hormone-based contraceptives with the study results. Details concerning subject selection are provided in the **Methods Supplement**.

Trial objectives, design, and treatments

The primary objective was to determine the relative bioavailabilities of digoxin, furosemide, metformin, and rosuvastatin given together as a test cocktail (T1) compared to when given individually as reference treatments. Secondary objectives were: to determine the relative bioavailabilities of digoxin, furosemide, and rosuvastatin administered as an additional test cocktail (T2) that consisted of a 2-fold higher dose of metformin compared to T1; to determine the relative bioavailabilities of digoxin, metformin, and rosuvastatin administered as an additional test cocktail (T3) that consisted of a 4-fold higher dose of furosemide compared to T1; to assess safety and tolerability.

This was a randomized, single-center, open-label, six-period, six-sequence crossover trial in healthy male subjects consisting of two parts with 12 subjects each. In both trial parts, the following treatments were administered according to one of the randomly assigned five treatment sequences:

- 0.25 mg digoxin (Lanicor, 0.25 mg film coated tablet, Teofarma, Italy),
- 5 mg furosemide given as 0.5 mL of a 10 mg/mL oral solution (Lasix liquidum, 5 mg, Sanofi-Aventis Deutschland, Germany)
- 500 mg metformin hydrochloride (Glucophage, 500 mg, film-coated tablet, Merck Serono, Germany)
- 10 mg rosuvastatin (Crestor, 10 mg film coated tablet, AstraZeneca, Germany)
- test cocktail T1 comprising the 4-fold combination of digoxin, furosemide, metformin, and rosuvastatin at the previously stated doses

Additionally, test cocktail T2 differed from T1 only in the dose of metformin hydrochloride, which was doubled to 1,000 mg. Part 2 was in general identical to part 1, with the exception that test cocktail T2 was replaced with test cocktail T3. T3 differed from T1 only in the dose of furosemide, which was increased 4-fold to 20 mg. Details of treatments and sequences are provided in **Table S4**. Treatments were separated by a washout period of at least 12 days.

Pharmacokinetics

Blood samples (4.0 mL) for the measurement of plasma concentrations of digoxin, furosemide, metformin, and rosuvastatin were taken using K_3EDTA as anticoagulant from a forearm vein of each subject before dosing and at 20 minutes, 40 minutes, 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, 10 hours, 12 hours, 24 hours, 36 hours, 48 hours, 72 hours, and 96 hours after dosing. The last three sampling timepoints were omitted after administration of furosemide alone. Urine fractions were collected for the determination of the analytes before dosing and at 0–4 hours, 4–8 hours, 8–12 hours, 12–24 hours, and 24–36 hours after dosing.

Pharmacokinetic parameters were calculated using standard noncompartmental methods with the software Phoenix WinNonlin (Professional, v. 6.3, Pharsight, Mountain View, CA). The primary endpoints were $AUC_{0\text{-}tz}$ (area under the plasma concentration–time curve up to the time of the last quantifiable plasma concentration t_z) and C_{max} (peak plasma concentration) for all four analytes. The secondary endpoint was $AUC_{0\text{-}\infty}$ (area under the curve extrapolated to infinite time).

Analytical methods

Drug concentrations were assessed by validated HPLC-MS/MS methods[31,32] using isotope-labeled internal standards [$^2H_3$]digoxin, [$^{13}C_6$]furosemide, [$^{13}C_1$,$^2H_4$]rosuvastatin, and [$^2H_6$]metformin. Details of the analytical methods are provided in the **Methods Supplement**.

Safety assessment

Safety and tolerability were evaluated based on adverse events (including clinically relevant findings from the physical examination), safety laboratory, 12-lead ECGs, and vital signs. Details concerning the safety assessments are provided in the **Methods Supplement**.

Statistical methods

The relative bioavailabilities of digoxin, furosemide, metformin, and rosuvastatin in the probe cocktails (test T1, T2, and T3) compared to the single substances (reference) were estimated from the ratios of the geometric means (test/reference) and their two-sided 90% CIs for the primary ($AUC_{0\text{-}tz}$, C_{max}) and secondary ($AUC_{0\text{-}\infty}$) endpoints. The statistical model used was an analysis of variance (ANOVA) on log-transformed endpoints including effects for sequence, subjects within sequences, period, and treatment. The effect "subjects within sequences" was considered random, whereas the other effects were

considered fixed. CIs were determined from the ANOVA residual error. No adjustment for a first-order carryover effect was included in the statistical analyses, as the occurrence of such an effect was highly unlikely due to the chosen study design and procedures. The statistical analyses were performed on the pooled pharmacokinetic set (all subjects from the treated set who provided at least one primary or secondary endpoint value in any period) using SAS (v. 9.2, by SAS Institute, Cary, NC).

The sample size calculation for this exploratory trial was not based on a power calculation but on the expected precision, defined as the ratio of upper to lower confidence limits of the 90% CI around the geometric mean ratio. For $N = 24$ evaluable subjects, the precision ranges for geometric coefficients of variation of 25% to 40% were from 1.35 to 1.61. The calculation was performed as described by Kupper and Hafner[33] using R version 2.14.2.

Additional Supporting Information may be found in the online version of this article.

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CONFLICT OF INTEREST

All authors are employees of Boehringer Ingelheim. K.H. was contracted by Boehringer Ingelheim as an external statistician.

AUTHOR CONTRIBUTIONS

The first two authors contributed equally to this article. P.S., T.G., and F.M. wrote the article; P.S., T.G., A.S., N.I., M.E.T., H.Z.G., T.E., and F.M. designed the research; P.S., T.G., K.H., and F.M. performed the research; P.S., T.G., K.H., M.W., D.G., and F.M. analyzed the data.

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