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Short communication

Verapamil decreases glucuronidase activity in the gut

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Abstract

The present investigation addressed the role of verapamil for oral pharmacokinetics of morphine-6-β-glucuronide (M6G). Male Sprague–Dawley rats received 62.5 mg kg^{-1} M6G-dihydrate orally w/wo pre-treatment with 70 mg kg^{-1} verapamil. Intravenous M6G (3.9 mg kg^{-1}) and oral morphine (52.7 mg kg^{-1} morphine–hydrochloride) were also employed. Oral bioavailability of M6G and the fraction of M6G deglucuronidated to morphine were estimated from areas under the plasma-concentration vs. time curves (AUC) of morphine and its glucuronides. As initial results pointed towards inhibition of glucuronidases by verapamil, its capability to specifically inhibit *E. coli* and/or rat intestinal β-glucuronidase was assessed using altered cleavage of the model substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). Oral bioavailability of M6G was 2.1%; 13% of oral M6G was deglucuronidated to morphine. Co-administration of verapamil did not increase the AUC of M6G. AUCs of morphine and morphine-3-glucuronide were smaller in the verapamil group than in controls. Verapamil co-administration decreased the fraction of M6G deglucuronidated to morphine to 4.6%. *In vitro* experiments provided evidence that verapamil inhibits β-glucuronidase from *E. coli* with an IC_{50} of 30 μM, whereas no inhibition of the rat β-glucuronidase from small intestine was seen. In conclusion, verapamil decreased intestinal deglucuronidation of M6G by inhibiting *E. coli* β-glucuronidase. This indicates that verapamil is not suited as P-gp inhibitor in experiments involving glucuronides. An increase in the intestinal absorption of M6G due to P-gp-inhibition was not observed at the verapamil dose studied.

Keywords: Morphine-6-β-glucuronide; Metabolism; Interaction; Rats; Glucuronidase; Intestinal pharmacokinetics

1. Introduction

Morphine-6-β-glucuronide (M6G) is an active [1] metabolite of morphine. High plasma levels of M6G have been related to side effects during morphine therapy in patients with impaired renal function [2]. In a series of experiments aimed at the development of a pharmacokinetic–pharmacodynamic model for M6G, we investigated possible factors important for the pharmacokinetics of M6G. The present study addressed the potential role of enterohepatic cycling of M6G on its pharmacokinetic behavior.

After its excretion into the bile, M6G may be reabsorbed as intact molecule or, after deglucuronidation in the intestine, as morphine. Typically, M6G has a bioavailability of less than 10% [3] making reabsorption of the intact molecule unlikely to be of importance for its pharmacokinetics. However, M6G appears to be a substrate of P-gp [4]. As shown for digoxin [5,6], cyclosporine and saquinavir [7], inhibition of P-gp is known to cause increased net uptake of its substrates from the intestine [8]. Thus, the present study was designed to investigate whether the P-gp inhibitor verapamil increased the bioavailability of M6G to a degree that might render enteral reabsorption to be an important part of M6G pharmacokinetics. Following a first series of experiments, we additionally investigated effects of verapamil on β-glucuronidase activity in the intestine because preliminary results indicated an inhibition of these enzymes in the presence of verapamil.

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Abbreviations: M6G, morphine-6-β-glucuronide; M3G, morphine-3-glucuronide; P-gp, P-glycoprotein; MUG, 4-methylumbelliferyl-β-D-glucuronide; MU, 4-methylumbelliferone; SL, saccharic acid 1,4-lactone; CYP, cytochrome P450; AUC, area under the curve; EDTA, ethylene diamine tetra-acetic acid; HPLC, high performance liquid chromatography; CL, clearance; $t_{1/2}$, half-life; C_{max}, observed maximum plasma concentration; t_{max}, time at that the maximum plasma concentration was observed; *F*, bioavailability.

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Table 1
Basic pharmacokinetic parameters of morphine and its glucuronides (median and range)[a]

	M6G	M6G + Ver	M6G	Morphine
Dose (nmol)	35462.1 (29075.8–38172.4)	35501.05 (34008.3–36215)	2020.85 (1911.5–2097.8)	40302.05 (39811.4–40932.8)
Route	Oral	Oral	Intravenous	Oral
AUCM3G (nmol L^{-1} hr)	5058.85 (4369.9–6255.2)	2076.95 (1258.6–2319.8)	125 (0–914.5)	44646.65 (37397.6–81696.1)
AUCM6G (nmol L^{-1} hr)	3007.15 (1919.8–3780.4)	2057.05 (1732.3–3275.2)	7415.25 (6837.5–10544.4)	0 (0–0)
AUCMorphine (nmol L^{-1} hr)	1074.8 (556.8–2369.1)	440.95 (194.1–1057.7)	0 (0–0)	10388.3 (7130.2–13987.7)
CL (mL min^{-1} kg^{-1})	–	–	18.2 (12.8-19.7)	–
$t_{1/2}$	–	–	0.63 (0.46–1.22)	–
C_{max} (nmol L^{-1})	760.5 (385.8–986.8)	833.4 (684.5–957.7)	–	3573.15 (3340.4–4303.2)
t_{max} (hr)	1 (1–4)	1 (1–2)	–	1 (1–1)
F (%)	2.35 (1.3–2.7)	1.6[b] (1.2–2.5)	100	–

[a] The AUCs are the areas under the observed plasma concentration versus time curves from time zero to the end of the sampling period. F: bioavailability in percent as estimated on the basis of i.v. data after administration of M6G alone.
[b] These values do not consider a possible different disposition of M6G when verapamil was co-administered.

2. Materials and methods

2.1. Study design and animals

Seventeen male Sprague–Dawley rats (weight 266 ± 22 g) received M6G, morphine and verapamil as listed in Table 1 after having fasted for 6 hr. More selective inhibitors than verapamil [9] were not believed to be necessary because the inhibition of cytochrome P450 (CYP) 3A by verapamil plays no role for formation or degradation of M6G. The rats were maintained in climate- and light-controlled rooms (24 ± 0.5°, 12/12 hr dark/light cycle). In all experiments the ethics guidelines for investigations in conscious animals were obeyed and the local Ethics Committee approved the procedures.

2.2. Pharmacokinetics of M6G, morphine and morphine-3-glucuronide (M3G)

M6G dehydrate with a chemical purity of more than 99.7% (Mundipharma GmbH) was dissolved in Ringer solution for intravenous administration and suspended in tylose slime for oral use. Verapamil (Sigma) was dissolved in 0.03 M phosphate buffer. Blood samples (75 μL) from a tail vein were collected into EDTA plastic tubes before and 1, 2, 4, 6, and 8 hr after drug administration. Plasma samples were stored at −25° pending analysis for the concentrations of M6G, morphine, and morphine-3-glucuronide (M3G) by HPLC [10] (limit of quantification 10 ng mL^{-1} for all analytes).

Basic pharmacokinetic parameters were assessed using standard non-compartmental techniques. The areas under the plasma concentration vs. time curve ($AUC_{0–last\ data\ point}$) were calculated using the linear/log trapezoidal. The median AUC of M6G obtained after intravenous and oral administration of M6G were used to calculate its oral bioavailability. The amount of morphine produced by deglucuronidation of M6G was estimated from the ratio of AUCs of the morphine after oral administration of M6G and the median AUC of morphine obtained after oral administration of morphine.To increase the reliability of this estimate, the latter calculations were repeated using the AUCs of M3G instead of those of morphine. Effects of verapamil on M6G bioavailability were analyzed by comparison of AUCs using Mann–Whitney-U-tests (α-level set at 0.05).

2.3. Inhibition of MUG cleavage by verapamil

Deep frozen samples from rat mucosa (derived from duodenum and jejunum) were homogenized using a Micro–Dismembrator S (B. Braun Biotech International) at 2300 rpm for 2 min. The frozen tissue powder was resuspended in 20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM pefabloc® (Roth) and protein concentration was determined by the method described by Lowry and collegues [11]. Because the MUG assay is widely accepted to be a reliable tool for the study of glucuronides, MUG was used rather than M6G. Incubations and analyses were performed as described previously [12]. In brief, 50 μL incubation mixtures contained 2.25 μg of total rat homogenate protein or 110 pg (0.001 units) of purified *E. coli* β-glucuronidase (Sigma), respectively. Assay buffers contained 0.2 mM MUG (Sigma) and 200 mM sodium acetate, pH 5.0; 10 mM EDTA; 0.1% [w/v] bovine serum albumin; 0.1% [v/v] Triton X-100 for rat β-glucuronidase or 50 mM sodium phosphate, pH 7.0; 150 mM sodium chloride; 10 mM EDTA; 0.1% [w/v] bovine serum albumin; 0.1% [v/v] Triton X-100 for *E. coli* β-glucuronidase, respectively. Verapamil or the specific glucuronidase inhibitor saccharic acid 1,4-lactone (Sigma) were preincubated with the incubation mixture for 10 min prior to addition of MUG. All incubations were carried out at 37° for 1 hr followed by adding 150 μL of 200 mM sodium carbonate to stop the enzymatic reaction. After centrifugation for 5 min at 13,000 rpm, supernatants were analyzed by HPLC using fluorescence detection (excitation 355 nm, emission 460 nm). Enzymatic activity was expressed as nmol 4-methylumbelliferone (MU) per mg (or μg) protein per hour. Interference of verapamil and the specific glucuronidase

inhibitor saccharic acid 1,4-lactone (SL) with cleavage of the β-glucuronidase substrate MUG was assessed at the respective pH optimum for rat (pH 5.0) and *E. coli* β-glucuronidase (pH 7.0), respectively. In addition, homogenates from rat jejunum and duodenum ($n = 3$ in each group) were compared for site-specific verapamil and SL-mediated β-glucuronidase inhibition. IC_{50} values were calculated with standard equations using GraphPad Prism (GraphPad Software Inc.).

3. Results and discussion

Basic pharmacokinetic parameters of M6G and morphine are listed in Table 1. Oral bioavailability of M6G was 2.3% (1.3–2.7%). This was even smaller than the previously reported value of 5–10% [3]. Plasma concentrations of M6G in the earlier part of the sampling period appeared to be higher in the verapamil than the control group but neither maximum concentrations nor the AUCs differed significantly between groups ($P > 0.5$). Thus, the present data do not support the hypothesis that P-gp inhibition by verapamil increases enteral recirculation of M6G to a clinically relevant degree. Higher dosing of verapamil was not considered because the dose used was well above the clinically relevant dose range. Thus, although it cannot be excluded that P-gp related interactions between M6G and verapamil are present at high experimental doses the clinical relevance of such an interaction would be questionable.

Thirteen percent (5.8–24.7%) of the oral M6G dose were deconjugated to morphine when AUC ratios of morphine after oral administration of M6G and morphine were used for the estimate. Using the M3G AUCs instead, the respective estimate was 12.6% (10.6–25.7%). AUCs of M3G were significantly smaller in the M6G plus verapamil group than that in the M6G alone group (Mann–Whitney U: 0, $P < 0.05$; Table 1). A similar tendency was observed for the AUCs of morphine (Table 1; Mann–Whitney U: 2, $P = 0.063$). When P-gp was inhibited by GF120918 [13] or verapamil [14] the decrease of morphine and M3G plasma concentrations contrasted to previous observations of increased plasma concentrations of morphine and M3G after systemic administration of morphine. Therefore, we hypothesized that the amount of morphine formed from M6G was smaller in the M6G plus verapamil than in the M6G alone group. When estimated from morphine AUCs, 4.6% (2–11%) of the M6G were deglucuronidated to morphine. The respective value estimated from the M3G AUCs was 5% (3–5.6%).

However, the appearance of morphine and M3G in plasma was delayed after administration of M6G. While this resulted in ascending plasma concentrations during most of the sampling period, the present pharmacokinetic data were insufficient to test the hypothesis of a decreased formation of morphine from M6G. Instead of repeating the pharmacokinetic experiments with a longer sampling period and additionally assessing the effects of P-gp inhibition on the disposition of morphine and its glucuronides, we decided to continue the study with *in vitro* experiments directly targeting the hypothesis. This had the additional advantage that we were able to establish whether the inhibition related to β-glucuronidase activity from rat intestinal mucosa or from bacteria [15,16]. We found that verapamil inhibited the bacterial enzyme with an IC_{50} value of 30 μM, whereas no inhibition of rat duodenal β-glucuronidase was seen (Fig. 1). There was no difference between duodenal and jejunal segments. In contrast to verapamil, SL inhibited *E. coli* β-glucuronidase as well as β-glucuronidase activity in homogenates from rat duodenum. Thus, the experiments showed that verapamil inhibited only *E. coli* β-glucuronidase. The IC_{50} of 30 μM is about five times below the KM value of 140 μM of the enzyme (Sperker, unpublished data).

In conclusion, the results suggest that intestinal reuptake of M6G is unlikely to be enhanced by P-gp inhibition to a degree that would render it a significant factor for the

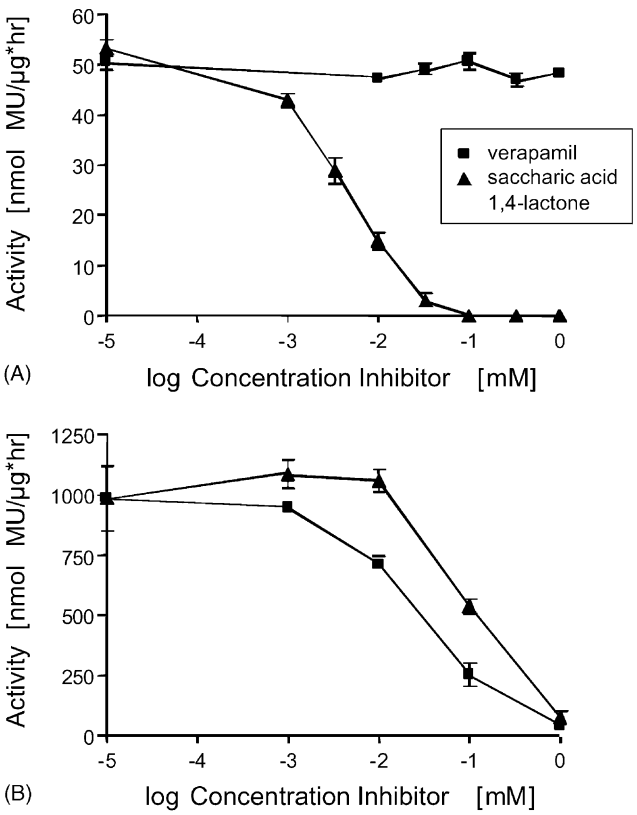

Fig. 1. In vitro effect of verapamil (■) and the specific inhibitor saccharic acid 1,4-lactone (▲) on β-glucuronidase activity. After preincubation with the indicated concentrations of inhibitor, MUG (4-methylumbelliferyl-β-D-glucuronide, final concentration 0.2 mM) was added to mixtures containing either 2.25 μg of total protein from rat duodenal homogenate (A) or 110 pg (0.001 units) of *E. coli* β-glucuronidase (B) followed by an incubation step at 37° for 1 hr. Released 4-methylumbelliferone (MU) was quantified by HPLC with fluorescence detection. Means ± maximum/minimum.

pharmacokinetics of M6G. In contrast, verapamil decreased the deglucuronidation of M6G by inhibition of *E. coli* β-glucuronidase. In addition, to the well-known inhibitory effects of verapamil on P-gp and on cytochrome P450 3A enzymes, the present data indicate that the inhibition of glucuronidases is another possibility for drug interactions with verapamil.

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