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Inhibition of the human organic anion transporter 1 by the caffeine metabolite 1-methylxanthine

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Abstract

Caffeine (1,3,7-trimethylxanthine) is daily and widely consumed in beverages and food and is mainly metabolized to 1,7-dimethylxanthine and 1-methylxanthine. Indirect clinical evidence suggests that 1-methylxanthine interacts with the organic anion transport system in the human kidney. In this study the effect of caffeine and its main metabolites on the human organic anion transporter 1 (hOAT1) was investigated using CHO cells overexpressing hOAT1. The uptake of 6-carboxyfluorescein into CHO^{hOAT} cells was significantly inhibited by $\geqslant$100 μM of 1-methylxanthine. Five hundred micromolar 1-methylxanthine was equieffective to 100 μM probenecid. In contrast, caffeine and 1,7-dimethylxanthine did not inhibit the transport of 6-carboxyfluorescein at concentrations up to 500 μM. In conclusion, the caffeine metabolite 1-methylxanthine inhibits the transport activity of hOAT1 in vitro. The central involvement of hOAT1 in the renal excretion of numerous drugs suggests that this inhibition may alter the pharmacokinetics of a series of clinically important drugs in humans.

Keywords: Organic anion transporter; Human; hOAT1; Caffeine; 1,7-Dimethylxanthine; 1-Methylxanthine; Inhibition; CHO cells

Caffeine (1,3,7-trimethylxanthine, 1,3,7-TMX) is one of the most widely consumed pharmacologically active substances with stimulating effects on the central nervous system [1]. Caffeine undergoes extensive hepatic metabolism resulting in the formation of paraxanthine (1,7-dimethylxanthine, 1,7-DMX) as the main metabolite (about 80% of caffeine). Paraxanthine is further metabolized to an unstable ring–open intermediate, which is stabilized by either an acetylation reaction to 5-acetylamino-6-formylamino-3-methyluracil (AFMU, about 15% of 1,7-DMX) or by internal rearrangement and the formation of 1-methylxanthine (1-MX, about 60% of 1,7-DMX). One-third of 1-MX is excreted unchanged into urine, whereas the rest undergoes hydroxylation to 1-methyluric acid [2].

Metabolic ratios of the molar concentrations of the caffeine metabolites in urine have been developed as surrogates for the individual activity of the enzymes responsible for the metabolism of caffeine and other drugs in humans [2]. Recently, co-administration of caffeine with either ibuprofen or probenecid (PBC) to healthy individuals has been shown to influence these ratios presumably by reducing the urinary concentrations of 1-MX [3]. Because both ibuprofen and PBC are inhibitors of organic anion transporters (OATs) in the human kidney [4], 1-MX is suspected to interact with the renal OAT system.

The human OAT-family (hOAT, SLC22A) comprises at least four distinct transport proteins hOAT1–hOAT4. The molecular structure of hOAT1 (SLC22A6) was identified as the *para*-aminohippurate (PAH)/dicarboxylate exchange transporter [5–9] located at the basolateral membrane of renal proximal tubule cells [10]. It is driven by an α-ketoglutarate gradient achieved via the

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Na^+/dicarboxylate co-transporter hNaDC3 [11] which itself is driven by a Na^+ gradient maintained by the Na^+/K^+-ATPase [12]. Due to its broad substrate spectrum, hOAT1 mediates the uptake of a wide variety of clinically important drugs into proximal tubule cells with subsequent excretion into urine. Among hOAT1 substrates are antibiotics like amoxicillin [13] and tetracycline [14], antiviral drugs like acyclovir, ganciclovir, zidovudine [15], cidofovir, adefovir [9], and oseltamivir [13], non-steroidal anti-inflammatory drugs (NSAID) such as salicylate, indomethacin, ibuprofen, and ketoprofen [16] as well as cytotoxic drugs like methotrexate [17].

Inhibition of renal tubular secretion of drugs mediated by hOAT1 may result in increased plasma drug concentrations, increased risk of drug toxicity, and hence reduced dose requirements [18]. Because 1-MX is suspected to be a substrate for the renal OAT system [3] and substrates of drug transporters may also act as inhibitors and because of the wide use of caffeine in most populations we investigated the inhibitory potency of caffeine and its main metabolites 1,7-DMX and 1-MX on the transport activity of hOAT1 in a stably transfected Chinese hamster ovary (CHO) cell line [19,20].

Materials and methods

Materials. Ham's F-12 with L-glutamine was purchased from PAA Laboratories GmbH (Parching, Austria), fetal calf serum (FCS), medium supplements, antibiotics, G-418, phosphate buffered saline (PBS), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonicacid (Hepes) were purchased from Invitrogen (Karlsruhe, Germany), fibronectin was from Roche Applied Science (Mannheim, Germany), NaCl, $MgSO_4$, dimethyl sulfoxide (DMSO), and Triton X-100 were from AppliChem (Darmstadt, Germany), and KCl, $CaCl_2$, $MgCl_2$, and glucose were from Merck. 6-Carboxyfluorescein was purchased from MobiTec (Göttingen, Germany), PBC, 1,3,7-TMX, 1,7-DMX, and 1-MX were from Sigma–Aldrich (Taufkirchen, Germany), tris(hydroxymethyl) aminomethane hydrochloride (Tris–HCl) was from Biomol (Hamburg, Germany), and 96-well microtiter plates and culturing bottles were from Nunc (Wiesbaden, Germany).

CHO^{pIRES} and CHO^{hOAT} cells. As an in vitro model for human hOAT1 we used CHO^{hOAT} cells, a cell line generated by stable transfection of hOAT1 cDNA into CHO cells. CHO^{pIRES}, containing only the empty pIRES neo expression vector (Stratagene, La Jolla, CA, USA), served as control [19,20]. Both cell lines were cultured in Ham's F-12 with L-glutamine supplemented with 10% heat inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.6 mg/ml G-418. For the transport assay cells were seeded on fibronectin coated microtiter plates in a density of 10,000 cells/well and cultured for 48 h without G-418.

Fluorescent transport assay. The transport assay was conducted according to a previously described procedure [19]. In brief, cells were washed once with PBS and incubated for 60 min at room temperature in Waymouth buffer (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.8 mM $MgSO_4$, 28 mM glucose, and 13 mM Hepes, pH 7.2) containing 4 μM of 6-carboxyfluorescein and the test compound (PBC, 1,3,7-TMX, 1,7,-DMX, or 1-MX) at different concentrations. Subsequently, transport was stopped by washing the cells three times with 200 μl precooled PBS. Cells were lysed with 200 μl of 0.4% Triton X-100 in 20 mM Tris–HCl, pH 9.0, for 30 min at room temperature. The lysates were transferred into 96-well plates and fluorescence was analyzed in a Fluoroskan Ascent fluorometer (Labsystems, Frankfurt, Germany) with 485 nm excitation and 535 nm emission filters. Each experiment was performed at least six times with $n = 8$ wells. Stock solutions of test compounds were prepared in DMSO. The fluorescence measured in the presence of each test compound was expressed as arbitrary units.

Statistical analysis. Data are expressed as means ± SD. p values were calculated by analysis of variance (ANOVA) with Dunnett's multiple comparison test for post hoc comparison in pairs with the corresponding control value. All statistical analyses were performed with GraphPad InStat, version 3.05, GraphPad Software (San Diego, CA, USA). A p value of $\leqslant 0.05$ was considered significant.

Results

The known hOAT inhibitor PBC inhibited the uptake of 6-carboxyfluorescein into CHO^{hOAT} cells in a concentration-dependent manner (Figs. 1–3). Similarly, the

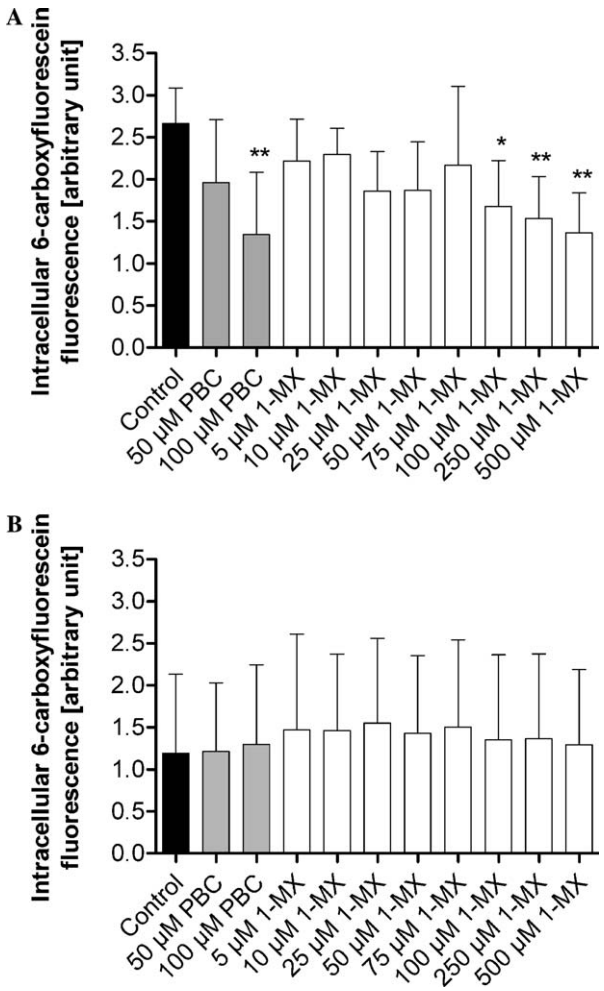

Fig. 1. Effect of PBC (grey bars) and 1-MX (white bars) on 6-carboxyfluorescein transport in CHO^{hOAT} cells (A) and CHO^{pIRES} (B) cells. Data are expressed as means ± SD with $n = 6$ experiments performed in octaplet. p values ($^*p < 0.05$ and $^{**}p < 0.01$) were determined by ANOVA with Dunnett's multiple comparison test for post hoc comparison of the results with the control without inhibitor (black bar).

caffeine metabolite 1-MX inhibited the uptake of 6-carboxyfluorescein in CHO^{hOAT} cells at concentrations $\geqslant$100 μM (Fig. 1) with 500 μM of 1-MX being equieffective to 100 μM PBC. Higher concentrations of 1-MX could not be tested due to limited solubility. In contrast, caffeine and 1,7-DMX had no inhibitory effect on the transport of 6-carboxyfluorescein at concentrations up to 500 μM (Figs. 2 and 3).

The uptake of 6-carboxyfluorescein in the control cell line CHO^{pIRES} accounted for approximately 50% of the uptake in CHO^{hOAT} cells without addition of an inhibitor. None of the compounds tested had any effect on the transport of 6-carboxyfluorescein in CHO^{pIRES} cells (Figs. 1–3). Moreover, none of the test compounds was quenching 6-carboxyfluorescein, no autofluorescence at the excitation wavelength used to measure 6-carboxyfluorescein (485 nm) was observed, and no self-quenching of 6-carboxyfluorescein was present.

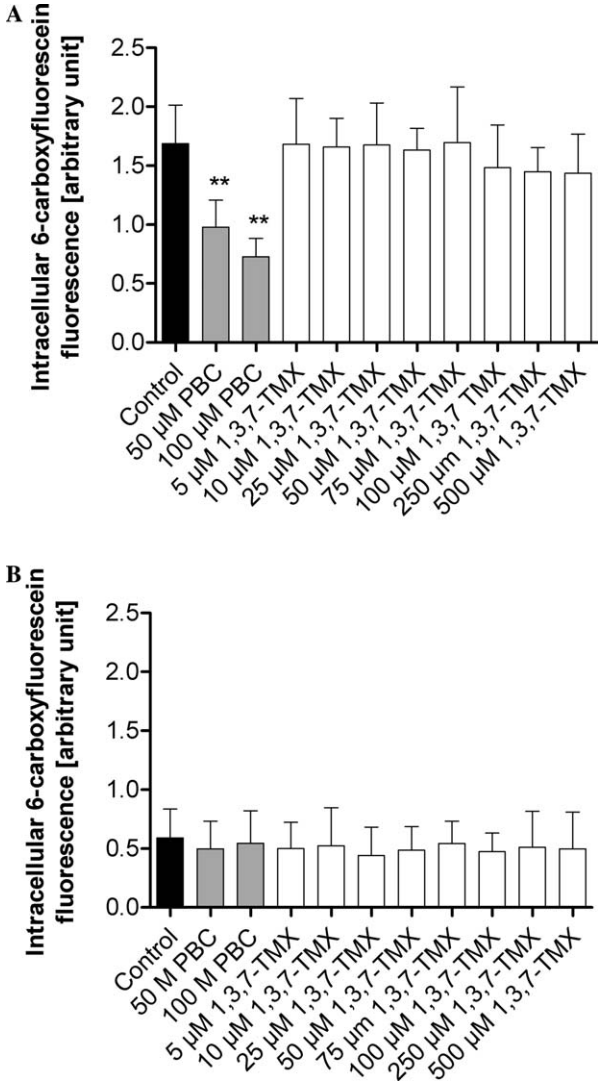

Fig. 2. Effect of PBC (grey bars) and 1,3,7-TMX (white bars) on 6-carboxyfluorescein transport in CHO^{hOAT} cells (A) and CHO^{pIRES} (B) cells. Data are expressed as means ± SD with $n = 6$ experiments performed in octaplet. p values (**$p < 0.01$) were determined by ANOVA with Dunnett's multiple comparison test for post hoc comparison of the results with the control without inhibitor (black bar).

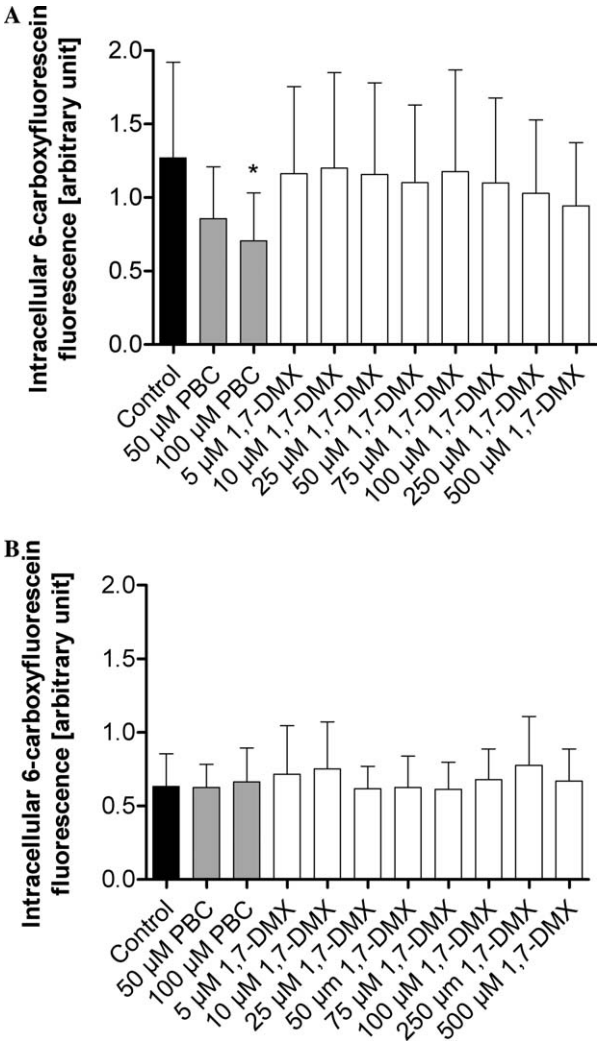

Fig. 3. Effect of PBC (grey bars) and 1,7-DMX (white bars) on 6-carboxyfluorescein transport in CHO^{hOAT} cells (A) and CHO^{pIRES} (B) cells. Data are expressed as means ± SD with $n = 10$ experiments performed in octaplet. p values (*$p < 0.05$) were determined by ANOVA with Dunnett's multiple comparison test for post hoc comparison of the results with the control without inhibitor (black bar).

Discussion

A wide variety of substances have been identified as inhibitors of transport mediated by hOAT1 with PBC being the prototypical non-specific inhibitor of OATs [4]. PBC has been shown to inhibit the hOAT1 mediated transport of 6-carboxyfluorescein, a recently established substrate with favorable specificity [19], and the classical substrate PAH in CHO cells with maximal inhibitory effect at concentrations of about 100 μM [19,20]. In the

present study, concentrations of 100 μM PBC reduced the uptake of 6-carboxyfluorescein in CHO^{hOAT} cells to levels similar to those in CHO^{pIRES} cells. Therefore, hOAT1-dependent transport was maximally inhibited by 100 μM PBC confirming data from previous reports [19,20].

Comparing the inhibitory efficacy of PBC with those of caffeine and its main metabolites the present study shows that caffeine and 1,7-DMX do not interfere with the transport activity of hOAT1 whereas 1-MX inhibited hOAT1 with approximately five times lower potency than PBC. These concentrations are in a similar order of magnitude as the concentrations of the diuretic furosemide (500 μM) needed for maximal inhibition of fluorescein uptake mediated by hOAT1 [21]. In contrast, the NSAID diclofenac led to maximal inhibition of hOAT1-dependent PAH transport at concentrations higher than 10 μM [16]. In regard to these data, 1-MX appears to be a moderate inhibitor of hOAT1 mediated transport of 6-carboxyfluorescein. The presence of an anionic moiety is one of the characteristics shared by most organic molecules interacting with OATs [22]. Nevertheless, substances lacking a typical anionic moiety like acyclovir, ganciclovir, or zidovudine have been shown to interact with hOAT1 both as substrates or inhibitors [15]. Even cationic molecules such as cimetidine are transported by hOAT1 indicating its wide substrate acceptance [23]. Therefore, the lack of an anionic moiety in the molecule of 1-MX does not argue against the observed inhibitory activity on the OAT system.

In addition to hOAT1, other members of the OAT-family have recently been characterized at the molecular level. hOAT3, another multispecific OAT acting as an exchange transporter in the human kidney [24], is located at the basolateral membrane of proximal tubule cells [10,25]. hOAT2 and hOAT4 are expressed only at low levels in human kidney cortex [10]. Whereas hOAT2 is also located at the basolateral membrane [26], hOAT4 is expressed at the apical membrane serving as an uptake transporter for the reabsorption of organic anions in exchange with dicarboxylates [27]. hOAT1 and hOAT3 exhibit overlapping substrate specificity [4] representing alternative pathways for the renal tubular secretion of drugs. This appears to be important especially for the secretion of drugs with a narrow therapeutic range like methotrexate [17]. Whether 1-MX also inhibits transport mediated by other OATs should be addressed because the impairment of bypass systems other than hOAT1 may further increase its interaction potential with co-administered OAT substrates. However, because hOAT1 plays a major role in the renal tubular secretion of organic substances [22], its inhibition by 1-MX might have an important clinical impact regardless of the influence of other transport proteins in the kidney.

The clinical relevance of an impaired renal excretion depends on the extent of inhibition, the therapeutic range of the inhibited substrate, and the proportion cleared by the kidneys. Hence, toxic adverse events caused by acute inhibition of renal tubular drug secretion [4] are particularly relevant for compounds such as methotrexate [18,28]. Because of the wide consumption of caffeine, an inhibitory effect of its metabolite 1-MX on renal tubular secretion mediated by hOAT1 might contribute to the pharmacokinetic variability of drugs such as methotrexate. Pharmacokinetic studies are needed to establish the clinical relevance of these findings.

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