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The role of OAT2 (SLC22A7) in the cyclic nucleotide biokinetics of human erythrocytes

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The present study was conducted to characterise the transporter(s) responsible for the uptake of cyclic nucleotides to human erythrocytes. Western blotting showed that hRBC expressed OAT2 (SLC22A7), but detection of OAT1 (SLC22A6), or OAT3 (SLC22A8) was not possible. Intact hRBC were employed to clarify the simultaneous cyclic nucleotide egression and uptake. Both these opposing processes were studied. The K_m-values for high affinity efflux was 3.5 ± 0.1 and 39.4 ± 5.7 μM for cGMP and cAMP, respectively. The respective values for low affinity efflux were 212 ± 11 and 339 ± 42 μM. The uptake was characterised with apparently low affinity and similar K_m-values for cGMP (2.2 mM) and cAMP (0.89 mM). Using an iterative approach in order to balance uptake with efflux, the predicted real K_m-values for uptake were 100–200 μM for cGMP and 50–150 μM for cAMP. The established OAT2-substrate indomethacin showed a competitive interaction with cyclic nucleotide uptake. Creatinine, also an OAT2 substrate, showed saturable uptake with a K_m of 854 ± 98 μM. Unexpectedly, co-incubation with cyclic nucleotides showed an uncompetitive inhibition. The observed K_m-values were 399 ± 44 and 259 ± 30 μM for creatinine, in the presence of cGMP and cAMP, respectively. Finally, the OAT1-substrate para-aminohippurate (PAH) showed some uptake (K_m-value of 2.0 ± 0.4 mM) but did not interact with cyclic nucleotide or indomethacin transport.

KEYWORDS
cAMP, cGMP, human erythrocytes, OAT2, SLC22A7

1 | INTRODUCTION

Early studies showed saturable cAMP uptake to hRBC (Holman, 1978; Thomas, King Jr, & Morrison, 1979) sensitive to temperature (Tsukamoto, Suyama, Germann, & Sonenberg, 1980). Later, a study of intact hRBC showed that cGMP uptake was temperature-sensitive and saturation of transport (Flo et al., 1995). These studies had in common an apparent K_m-value of cyclic nucleotide uptake of 3–5 mM. However, the membrane transporter was not identified. Cellular efflux of cyclic nucleotides required energy (Hamet, Pang, & Tremblay, 1989; Rindler, Bashor, Spitzer, & Saier Jr, 1978). In contrast to the cellular uptake, the proteins responsible for efflux were idetified as MRP4 (ABCC4) for cAMP (Chen, Lee, & Kruh, 2001) and MRP5 (ABCC5) for cGMP (Jedlitschky, Burchell, & Keppler, 2000). The respective K_m-values for cellular efflux were ≈ 40 μM for cAMP and ≈2 μM for cGMP. These proteins were also expressed in hRBC (Boadu & Sager, 2004; Jedlitschky et al., 2000; Klokouzas, Wu, van Veen, Barrand, & Hladky, 2003; Wu, Klokouzas, Hladky, Ambudkar, & Barrand, 2005). Later,

hOAT2 has come into focus with regard to cellular uptake of cGMP (Cropp et al., 2008; Marada et al., 2015). The affinity (K_m-values of 90–100 μM) reported for hOAT2 transfected HEK293 cells was clearly higher than that calculated based on a previous study on uptake to intact hRBC (K_m-values of 3–5 mM) (Flo et al., 1995).

As far as we know, expression of hOATs has not been reported previously for hRBC. This study was conducted to show whether hOAT2 is the transporter responsible for uptake of cyclic nucleotides to hRBC. In addition to Western blotting, we used pilot substrates for hOAT2 to reveal transport and competition for the cGMP uptake transporter. Indomethacin and creatinine were included. Indomethacin is a potent inhibitor of cGMP uptake in hOAT2 transfected HEK293 cells (Henjakovic, Hagos, Krick, Burckhardt, & Burckhardt, 2015; Hotchkiss, Berrigan, & Pelis, 2015), and of creatinine transported by hOAT2 (Shen et al., 2015).

2 | MATERIALS AND METHODS

2.1 | Western blot

Fresh human EDTA blood was obtained from healthy donors (Laboratory medicine, University hospital North Norway). All subsequent steps after collecting fresh human EDTA blood were performed at 4 °C. After the first centrifugation (2,300*g* for 10 min), plasma, and buffy coat were removed. The red blood cells were washed three times with 5 mM Tris-HCl, 113 mM KCl, pH 8.1 (centrifugation at 1,000*g*). The cells were lysed in 10 volumes of 5 mM Tris-HCl, 0.5 mM EGTA, and 4 mM KCl (pH 8.1) and centrifuged (20,000*g* for 20 min). The ghosts were washed in 1.47 mM KH_2PO_4, 81 mM, and 140 mM KCl (pH 7.6) until they appeared milky white. Afterwards the membranes were washed with RIPA Lysis and Extraction buffer (Thermo Scientific, Rockford, IL). Membrane lysates were sonicated and insoluble materials were removed by centrifugation at 15,000*g* for 10 min at 4 °C. Protein concentrations were determined using NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). For Western blotting experiments 50 μg of total protein lysate were applied per lane. Proteins were separated on NuPAGE Novex 4–12% Bis-Tris Gel (Invitrogen by Thermo Scientific, Carlsbad, CA). After SDS polyacrylamide gel electrophoresis the proteins were transferred to Immobilon—P polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA). The membrane was blocked with 5% (w/v) non-fat dried skimmed milk powder in TBST, pH 7.5 (25 mM Tris, 150 mM NaCl, and 0.05% Tween-20 (Thermo Scientific, Rockford, IL) for 1 hr at room temperature. The membranes were incubated with appropriate concentrations of primary antibodies (diluted in blocking solution) overnight at 4 °C. Goat polyclonal OAT1 (N-13) 1:200, rabbit polyclonal OAT2 (G-18) 1:200, rabbit polyclonal OAT3 (H-44) 1:200, and mouse monoclonal Na^+/K^+-ATPase α1 (F-2) 1:200 from Santa Cruz Biotechnology Inc., Santa Cruz, CA. The membranes were washed with TBST three times. Horseradish peroxidase saline-conjugated secondary antibodies were diluted in blocking solution (goat anti-rabbit IgG 1:5000, donkey anti-goat IgG 1:5000 obtained from Santa Cruz Biotechnology Inc., and goat anti-mouse Ig 1:1000 obtained from BD Biosciences (San Diego, CA) were added for incubation at room temperature for 1 hr. The membranes were washed two times with TBST (25 mM Tris, 150 mM NaCl, and 0.05% Tween-20® (Thermo Scientific, Rockford, IL) pH 7.5 and two times with 10 mM Tris-HCl, 10 mM NaCl, 1 mM $MgCl_2$. After washing, each protein were detected with Super-signal West Dura Extended Duration Substrate (Thermofisher Scientific, Rockford, IL) and electrochemiluminescence (Image Quant, LAS 4000, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for imaging.

2.2 | Transport experiments

Fresh human EDTA blood was obtained from healthy donors (Laboratory medicine, University Hospital North Norway). The blood cells were separated from plasma and washed with KRPB (122 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 15.8 mM Na_2HPO_4, and 1.3 mM $CaCl_2$, pH 7.4) with 10 mM D-glucose. Plasma and buffy coat were removed. The red cells were washed three more times in KRPB/10 mM D-glucose. In pilot experiments leukocytes and platelets were removed completely with a column filled with α-cellulose fibers (Sigma–Aldrich, Schnelldorf, Germany) basically according to (Beutler, West, & Blume, 1976). However, we did not detect any differences on transport between these two methods and chose the simplest procedure as routine method. Washed hRBC were routinely suspended in KRPB (pH 7.4)/10 mM D-glucose at a haematocrit of 0.4. Haematocrit was controlled in each experiment. The uptake was initiated by the addition of the substance(s) to be characterized. After incubation for 60 min (or indicated time) at 37 °C, the uptake was stopped by placing the tubes in ice water. The incubation medium was aspirated and the substrate concentrations were determined. The cells were washed four times with KRPB/10 mM D-glucose (2–4 °C). In some of the experiments, the concentrations were determined in the suspension as whole. The efflux of cGMP and cAMP was determined after loading hRBC, and removing the excess cyclic nucleotides, as described above, and initiated by resuspending the cells in KRPB/10 mM D-glucose at 37 °C. After 120 min the efflux process was terminated with placing the tubes in ice water.

2.3 | Assays with HPLC

In some experiments a HPLC method was used to determine intra- and extracellular concentrations of cGMP, cAMP, and PAH (Sigma Aldrich, Schnelldorf, Germany). The internal standard was 8-Br-cGMP (Sigma Aldrich, Schnelldorf, Germany). Samples of incubation medium and of hRBC suspensions were frozen immediately and stored at −22 °C awaiting analysis. After thawing, the samples (200 μl) were mixed with 2 M perchloric acid (200 μl) and internal standard (50 μl). The mixture was neutralised with $KOH/KHCO_3$ (1:1) to achieve pH 7.0. The concentrations were determined with HPLC/UV detection (254 nm). The equipment comprised of Dionex P680 pump and Ultimate 8000 UV detector (Thermo Fisher Scientific, Inc., Sunnyvale, CA), a Waters Novapak 25 cm C18 column with a Waters Symmetry guard column (Waters, Oslo, Norway). The mobile phase comprised of 15% (v/v) methanol and 85% (v/v), 20 mM K_2HPO_4/KH_2PO_4, pH 6.6. The flow rate was 1.2 ml/min at ambient temperature. The respective peaks appeared after 2.6 min for PAH, 3.2 min for cGMP, 5.7 min for cAMP, and 6.6 min for the internal standard 8-Br-cGMP.

2.4 | Assays with LC-MS/MS

The following chemicals and solutions were employed: LC-MS grade methanol, acetonitrile, and formic acid were purchased from Fluka (Sigma–Aldrich, St. Louis, MO). Ultrapure water (18.2 MΩ-cm) was obtained from a Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France). Cyclic GMP, cAMP and creatinine was purchased from Sigma-Aldrich (Steinheim, Germany). Creatinine-d3, [$^{13}C_5$]-cGMP, and [$^{13}C_5$]-cAMP were obtained from Toronto Research Chemicals Inc. (Ontario, Canada). All stock solutions were separately dissolved in water at a concentration of 5 mM cAMP and 5 mM cGMP stored at −70 °C, and 100 mM creatinine stored at 4 °C. The isotopes were dissolved in water, [$^{13}C_5$]-cAMP with 0,01% methanol, and [$^{13}C_5$]-cGMP with 0.1% methanol, at a concentration of 150 nM. Creatinine-d3 was dissolved in water 0.1% methanol at a concentration of 150 nM. For the quantification of cAMP, cGMP, and creatinine a six-point standard curve was employed (10 nM–1,000 μM).

The preparation of cells and incubation was performed as described above. After incubation 50 μl of sample/standard was mixed with 200 μl of 0.1 M $ZnSO_4$, and then 500 μl acetonitrile. The mixture was centrifuged at 13,400*g* for 2 min at 4 °C. The cGMP and cAMP samples/standards were diluted 1:10 with methanol/water (1:1). The creatinine samples/standards were diluted 1:100 with methanol/water (1:1). The respective internal standard (30 nM) were included in both the sample/standard.

The analytical equipment comprised of Waters Acquity™ I-class UPLC system with an autosampler and a binary solvent delivery system (Waters, Milford, MA) interfaced to Waters Xevo TQ-S benchtop tandem quadrupole mass spectrometer (Waters, Manchester, UK) operated under positive electrospray ionization (ESI) conditions. Chromatographic separations of cAMP and cGMP were performed on a 2.1 × 100 mm Waters Acquity Amide BEH column, 100Å, 1.7 μm maintained at 45 °C. The sample injection volume was 4 μl. Mobile phase A consisted of 0.1% formic acid in water; mobile phase B was 0.1% formic acid in acetonitril. The following gradient elution profile was applied at a flow rate of 0.6 ml/min: 0.00 min: 100% B, 1 min: 98% B, 2 min: 70% B, 2.5 min: 50% B, 4 min: 50% A, 4.01 min: 100% B. The chromatography for creatinine was performed on a 2.1 × 100 mm Waters Acquity HSS T3 Column, 100Å, 1.8 μm maintained at 50 °C. The injection volume was set to 0.8 μl. Eluent A consisted of 0.1% formic acid in water; eluent B was 0.1% formic acid in methanol. Isocratic elution was performed with 40% B. The flow rate was 0.45 ml/min and the column temperature was kept at 50 °C.

2.5 | Assay with radioactivity

The following radiolabelled substances were employed: [^{3}H]-PAH (sp. act. 4.04 C_i/mmol, Perkin Elmer, Inc., Boston, MA) and [^{3}H]-indomethacin (5C_i/mmol, Montebello Diagnostics AS, Oslo, Norway). Unlabelled PAH and indomethacin were obtained from Sigma Aldrich (Schnelldorf, Germany). The desired total concentration range was obtained by a mixture of labeled and various unlabeled substance. The radioactivity was quantified (Packard1900 TR liquid scintillation analyzer), after addition of scintillation fluid (Ultima Gold^{XR}, Packard, Groningen, The Netherlands).

2.6 | Indirect determination of intracellular concentrations

Since it is very difficult to pipette an exact volume of packed hRBC an indirect method was employed to control cell concentrations in some of the experiments. This method is based on haematocrit (Hct), total substrate concentration in cell suspension ([Total]) and substrate concentration in incubation medium ([KRPB]): [hRBC] = [Total] × (1/Hct) − [KRPB] × ((1/Hct)-1).

2.7 | Estimates of real K_m-values for cyclic nucleotide uptake

OAT2 has been reported to transport cyclic nucleotides (Cropp et al., 2008; Marada et al., 2015). Since hRBC have enzymes for synthesis and degradation, in addition to effective efflux systems for cyclic nucleotides μM (Orvoll, Lysaa, Ravna, & Sager, 2013; Sager, Orbo, Pettersen, & Kjørstad, 1996), it is likely that kinetic parameters like K_m-values for OAT2-mediated uptake were underestimated. Kinetic parameters (K_m and V_{max}) have not been reported for hRBC guanyl cyclase and adenyl cyclase, or cnPDEs (cyclic nucleotide phosphodiesterases). Extracellular (plasma) concentrations of 13.6 nM and 4.1 nM have been reported for cAMP and cGMP, respectively (Peracchi et al., 1983). Assuming that the physiologic extracellular levels are 10–20 μM, the intracellular levels 0.1–10 μM, and that uptake balances efflux at steady state, an iterative method was employed to estimate real K_m-values for OAT2 uptake of cyclic nucleotides. The kinetic parameters for MRP5 high and low affinity efflux and MRP4 low affinity efflux and a range of possible K_m-values for cGMP uptake were employed to obtain the best fit. A corresponding approach was employed for OAT2 uptake of cAMP with kinetic parameters for MRP4 high and low affinity efflux and MRP5 low affinity efflux.

2.8 | Analysis of data

The concentration saturation data were analyzed according to Lineweaver and Burk (1934).

3 | RESULTS

3.1 | Expression of organic anion transporters

Western blots were performed to detect members of the OAT-family in hRBC membranes. Figure 1 (panel A) shows the expression of OAT2 in hRBC from six healthy individuals, appearing at approximately 50 kD. The positive OAT2 control from Jurkat cells showed somewhat higher molecular weight whereas the positive control for Na/K-ATPase appeared with same molecular weight. In contrast, OAT1 (panel B) and OAT3 (panel C) were not detected in hRBC.

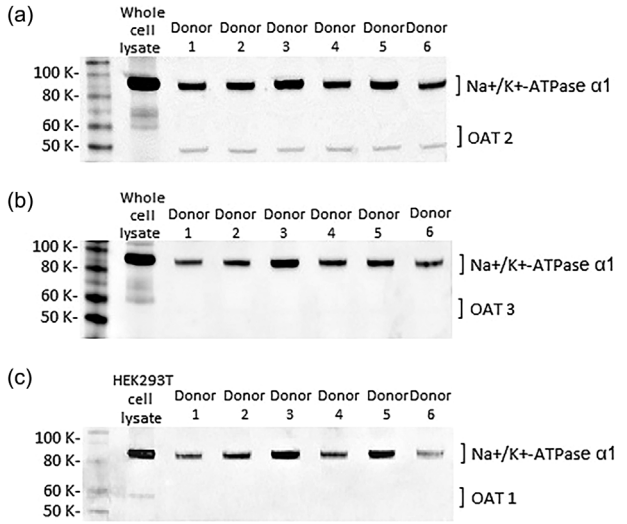

FIGURE 1 Western blotting of OAT1, OAT2, and OAT3 expression in hRBC membranes. Panel A: Positive control, OAT2 in Jurkat whole cell lysate and OAT-2 expression in hRBC membranes from three female donor (donor 1–3; applied 50 μg of total protein per lane), and three male donor (donor 4–6; applied 50 μg of total protein per lane). Panel B: Positive control, OAT1 in HEK 293T cell over-expression lysate and absence of OAT1 expression in hRBC membranes from three female donor (donor 1–3; applied 50 μg of total protein per lane), and three male donor (donor 4–6: applied 50 μg of total protein per lane). Panel C: Positive control, OAT3 in Jurkat whole cell lysate and absence of OAT 3 expression in hRBC membranes from three female donor (donor 1–3; applied 50 μg of total protein per lane), and three male donor (donor 4–6; applied 50 μg of total protein per lane). Na/K-ATPase α_1 was used as an internal control

3.2 | Efflux of cyclic nucleotides

In an intact cell model, cyclic nucleotide uptake and efflux occur simultaneously. This may reduce the intracellular levels achieved by uptake and hamper the interpretation of data. Due to this, the efflux of cyclic nucleotides was characterized for intact hRBC, in addition to uptake. The extrusion of cGMP and cAMP was composed of a high and low affinity component (Table 1).

3.3 | Uptake of cyclic nucleotides

Figure 2 (panel A) shows the time course of cyclic nucleotide uptake. Temperature equilibration from 4 °C to 37 °C caused lag-phases of 10–20 min. Afterwards, a linear uptake of both nucleotides was observed. During the incubation period of 60 min no saturation occurred. The uptake of cAMP appeared to be approximately twice as high as for cGMP.

Figure 2 (panel B) demonstrates the concentration-dependency of the cyclic nucleotide uptake after 60 min at 37 °C. Table 2 shows the values of K_m and V_{max} obtained for cGMP and cAMP. The affinities were low for both, but apparently highest for cAMP. However, the K_m-values might have been underestimated because the co-existing functional efflux pumps.

3.4 | Estimates of real K_m-values for cyclic nucleotide uptake

To estimate real values, an iterative approach (described in methods) was employed and gave a best fit for following K_m-values; 100–200 μM for cGMP and 50–150 μM for cAMP. This suggests markedly higher affinities for cyclic nucleotide uptake than obtained in the present experimental setting due to simultaneous cyclic nucleotide elimination.

3.5 | Creatinine uptake

Creatinine is now recognized as a substrate for OAT2 (Shen et al., 2015). Figure 3 shows the time course of creatinine uptake. Washed hRBC were incubated with creatinine at 37 °C in periods up to 120 min. A linear uptake occurred between 0 and 30 min. A plateau (≈40 nmol/10^9 cells) was seen after 60 min.

Additional experiments were performed to characterize the relationship between creatinine concentration and uptake. The cells were incubated with creatinine for 60 min at 37 °C. A classical Michaelis–Menten relationship was evident (data not shown). Table 3 shows that co-incubation with cGMP and cAMP appeared to decrease both K_m and V_{max}. In classical enzyme kinetics, this is described as uncompetitive inhibition.

3.6 | Uptake of indomethacin

Indomethacin is a potent inhibitor of OAT-2 transfected HEK-cell transport with reported IC_{50}-values from 2.1 μM (Shen et al., 2015). In the present study, indomethacin was tested for its ability to be a substrate for hRBC OAT2. A range of unlabelled concentrations (5–

TABLE 1 The cells were loaded with cyclic nucleotides and the efflux initiated at 37 °C and terminated after 120 min

	High affinity		Low affinity	
	K_m (μM)	V_{max}[a]	K_m (μM)	V_{max}[a]
cGMP (n = 4)	3.5 ± 0.1	0.146 ± 0.044	212 ± 11	0.541 ± 0.134
cAMP (n = 5)	39.4 ± 5.7	0.222 ± 0.074	339 ± 42	0.947 ± 0.364

The levels of cGMP and cAMP were determined with a HPLC assay (described in methods). The results are presented as mean value ± SD and the number of time-independent experiments in parenthesis.

[a]pmol per 10^9 cells per min.

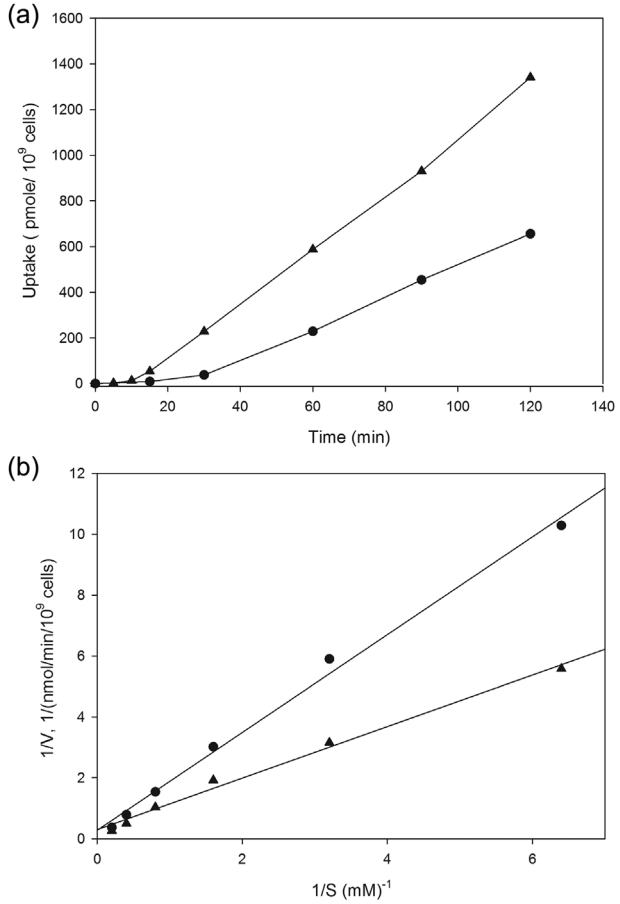

FIGURE 2 Panel A shows uptake of cyclic nucleotides during an incubation period of 120 min. Washed hRBC were incubated with 10 μM cyclic nucleotide at 37 °C. The intracellular concentrations were determined with LC-MS/MS as described in methods. Three time-independent experiments were performed with cGMP (● – ●) and cAMP (▲-▲). Panel B shows a concentration-dependent uptake of cyclic nucleotides from the buffered medium (160–5,000 μM) after 60 min at 37 °C, presented in a Lineweaver–Burk plot. Cyclic nucleotide concentrations were determined with HPLC as described in methods. Uptake of cGMP ($n = 4$) (● – ●) and cAMP ($n = 5$) (▲- ▲)

500 μM) in presence of tracer amounts of [^{3}H]-indomethacin were employed to determine uptake after 60 min at 37 °C. The uptake process showed a classical saturation kinetics. Table 4 shows that indomethacin, apparently, was taken up with moderate affinity

TABLE 2 Observed K_m and V_{max} for the uptake of cGMP and cAMP after 60 min at 37 °C

	cGMP ($n = 4$)	cAMP ($n = 5$)
K_m (mM)	2.2 ± 0.5	0.89 ± 0.12
V_{max} (pmol/min/10^9 cells)	4.4 ± 0.5	3.7 ± 0.8

Cyclic nucleotide concentrations were determined with HPLC assay. The results are presented as mean value ± SD and the number of time-independent experiments in parenthesis.

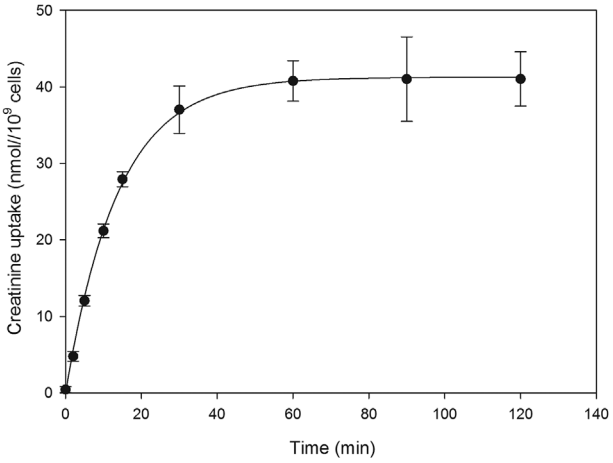

FIGURE 3 Washed hRBC were incubated with 500 μM creatinine at 37 °C for periods up to 120 min. Intracellular concentrations were determined with LC-MS/MS. The results from three time-independent experiments (each in duplicate) are presented as mean ± SD, $n = 3$

($K_m \approx 500$ μM). The co-incubation with cGMP and cAMP gave classical competitive inhibition with five times higher K_m-value (≈2.5 mM).

3.7 | Uptake of PAH

The uptake of [^{3}H]-PAH to hRBC showed a temperature sensitive transport system. The uptake after 120 min was reduced to 22.1 ± 1.8% at 22 °C and 4.5 ± 0.9% at 4 °C (mean ± SD, $n = 3$) compared to the transport observed at 37 °C. The saturation kinetics was characterized for concentrations between 50 μM and 1 mM at 37 °C with a HPLC assay and showed classical Michaelis-Mentens kinetics (data not shown). Analysis of data resulted in a K_m-value of 2.0 ± 0.4 mM with a V_{max} of 4.6 ± 0.7 nmol/10^9 cells/min (mean ± SD, $n = 3$). In separate experiments, the uptake of cyclic nucleotides (1,000 μM) were determined (after 15, 30, 60, 90, and 120 min) in the presence of five concentrations (100–1000 μM) of PAH. The K_m-value of indomethacin was not influenced by PAH.

TABLE 3 Creatinine uptake in absence and the presence of cyclic nucleotides

	K_m (μM)	V_{max}[a]
Creatinine	854 ± 98 ($n = 3$)	1.7 ± 0.15 ($n = 3$)
Creatinine + cGMP	399 ± 44 ($n = 3$)	1.0 ± 0.14 ($n = 3$)
Creatinine + cAMP	259 ± 30 ($n = 3$)	0.8 ± 0.03 ($n = 3$)

Nine creatinine concentrations (20–5000 μM) were incubated for 60 min at 37 °C in the absence and presence of 500 μM cGMP or 500 μM cAMP. Intracellular creatinine concentrations were obtained with LC-MS/MS as described in methods. The results are presented as mean value ± SD and the number of time-independent experiments in parenthesis.
[a]μmol/10^9 cells/min.

TABLE 4 The transport of indomethacin, a mixture of [^{3}H]-indomethacin and unlabelled indomethacin (5–500 μM), was determined at 37 °C after 60 min incubation in the absence or in presence of cGMP (500 μM) or cAMP (500 μM) or PAH (500 μM)

Competitor	K_m (mM)	V_{max}b
None (n = 5)	0.475 ± 0.08	2.6 ± 0.5
cGMP (n = 3)	2.3 ± 0.4a	2.5 ± 0.4
cAMP (n = 3)	2.4 ± 0.2a	2.6 ± 0.5
PAH (n = 3)	0.502 ± 0.1a	2.1 ± 0.4

The resulting K_m–values without inhibitor and apparent K_m–values with inhibitors are given as mean value ± SD and number of time-independent experiments.

[a]Apparent K_m for Indomethacin in presence of potential inhibitors.

[b]Indomethacin transport nmol/10^9 cells/min.

4 | DISCUSSION

The human OAT- transporter family comprises eight members (including the URAT1 transporter) (Burckhardt, 2012; Nigam et al., 2015). These transporters have been extensively studied with respect to renal excretion and hepatic biotransformation of endogenous and exogenous substances but also their role in distribution, like transport across the blood brain barrier (Koepsell, 2013). Among the OAT family, only hOAT2 has ubiquitous expression pattern with expression in many tissues such as choriod plexus, liver, placenta, skeletal muscle, and kidney (Burckhardt & Burckhardt, 2011). The present observation of OAT2 expression in hRBC cell membranes supports the idea of a generalized tissue distribution. The OAT2-tv1 protein (546 aa) is localized to the plasma membrane (Cropp et al., 2008; Hotchkiss et al., 2015). In our experiments, the positive control from Jurkat cells appeared to have slightly higher molecular weight compared to hRBC OAT2. The large extracellular loop found between TMD1 and TMD2 carries N-glycosylation sites (Tanaka, Xu, Zhou, & You, 2004). Treatment with endoglycosidase did not modify the protein but caused a marked reduction in molecular weight (≈40%) (Geng et al., 1999). Deglycosylation of membrane proteins during the last part of the hRBC's life cycle (Huang et al., 2011) may explain the lower molecular weight observed for hRBC OAT2.

The intracellular signalling of cGMP and cAMP is terminated by cnPDE activity (Azevedo et al., 2014) and ATP-dependent efflux pumps (Chen et al., 2001; Jedlitschky et al., 2000). Energy-requiring cAMP-export was described in cell physiologic studies four decades ago (Rindler et al., 1978). In hRBC the basal intracellular levels of cyclic nucleotides are low; ≈ 1.4 pmol/10^{10} cell for cGMP (Conran et al., 2004) and cAMP: ≈ 2 pmol/10^{10} cell (Hanson et al., 2008). Enzymes producing cGMP and cAMP are present in hRBC. Both soluble and particulate guanyl cyclase exist (Cortese-Krott et al., 2018; Petrov & Lijnen, 1996), in addition to adenyl cyclase (Rodan, Rodan, & Sha'afi, 1976). Also the uptake of cyclic nucleotides from the extracellular compartment elevates intracellular levels, described decades ago in cell physiologic studies (Holman, 1978; Thomas et al., 1979). At that time, the complexity of cellular elimination of cyclic nucleotides was unknown. A membrane bound cnPDE that hydrolysed cAMP was reported four decades ago (Suzuki, Terao, & Osawa, 1980). Except for this, knowledge about hRBC cnPDEs has until recently virtually been absent. The existence of other hRBC cnPDEs have been reported (specificity given in parenthesis): PDE2A (cAMP = cGMP), PDE3A (cAMP>cGMP), PDE3B (cAMP>cGMP) and PDE4A (cAMP>>cGMP) and PDE5A (cGMP>>cAMP) (Adderley et al., 2009, 2011; Hanson et al., 2008).

MRP5 was identified as a transporter for cGMP (Jedlitschky et al., 2000). An ATP-dependent cGMP pump had been described for hRBC (Sager et al., 1996) and these cells expressed MRP5 (Boadu & Sager, 2004; Jedlitschky et al., 2000; Wu, Woodcock, Hladky, & Barrand, 2005). MRP4 was recognised as a cAMP efflux pump (Chen et al., 2001) and this protein was expressed in hRBC (Klokouzas et al., 2003; Wu, Woodcock, et al., 2005). In IOVs from hRBC, the K_m-values of selective high affinity cGMP efflux (2–4 μM) is 10–20 times lower than that of selective high affinity cAMP efflux (35 μM) (Orvoll et al., 2013; Sager et al., 1996). The low affinity component of both pumps was nonselective with similar affinities (Jedlitschky et al., 2000; Orvoll et al., 2013; Schultz, Vaskinn, Kildalsen, & Sager, 1998). The results obtained in the present study of intact hRBC cyclic nucleotide efflux were in were in close agreement to the data from IOVs reported earlier uptake (Orvoll et al., 2013; Sager et al., 1996).

The model with intact cells represents a challenge when simultaneous uptake and extrusion exist. The available data on kinetic properties was used to predict more real K_m-values for OAT2-mediated cyclic nucleotide uptake. The affinities observed experimentally (K_m-values of 2–4 mM) were similar to those previously reported (Holman, 1978; Thomas et al., 1979) but the simultaneous reduction in intracellular concentrations of both cyclic nucleotides due to efflux, results in an underestimation of affinities for uptake. The P-glycoprotein (ABCB1) transport in $CaCO_2$ cells represented a similar challenge (Tachibana et al., 2010). In the present study the estimated real K_m-value of OAT2 was 50–150 μM for cAMP and 100–200 μM for cGMP. The present K_m-value for cGMP transport is in close correspondence with the values reported for HEK 293 cells lines with overexpression of OAT2-tv1, 88 μM (Cropp et al., 2008) and 101 μM (Marada et al., 2015). However, the impact of the other biokinetic components (synthesis and hydrolysis) remains to be answered. From a pharmacologic point of view, selective inhibitors may clarify this.

Creatinine is actively secreted to the preurine in addition to glomerulus filtration (Levey, Perrone, & Madias, 1988). OCT1 and OCT2 have been shown to be highly expressed in kidney from mice (Alnouti, Petrick, & Klaassen, 2006). Among hOCT1, hOCT2, and hOCT3, only hOCT2 caused a significant creatinine uptake when stably expressed in HEK293 cells (Urakami, Kimura, Okuda, & Inui, 2004). In vivo measurements performed in wild-type and Oct1/2 (−/−) mice further demonstrated the importance of OCTs for creatinine secretion (Ciarimboli et al., 2012). Stable expression of hOCT2 gave a significant accumulation of creatinine in HEK293 cells (Ciarimboli et al., 2012). A role of OATs was also demonstrated in a study of knockout mice wherein the renal creatinine clearance was much higher than that attributable to GFR in wild-type mice but not in mice lacking OAT1 (Oat1 −/−) and OAT3 (Oat3 −/−) (Vallon et al., 2012). These results are

consistent with OAT3 as creatinine transporter and that OAT1 might contribute to renal creatinine secretion in mice. Recent reports proves that hOATs, and especially hOAT2, contributes to creatinine transport (Shen et al., 2015). The fact that creatinine is a tautomer explains the observations of creatinine transport by both anion and cation transporters.

Creatine is a waste product from the cells' energy turnover and renal secretion represents an effective elimination from the body. The vectorial transport of creatinine is secured by P-glycoprotein and MRP2 at the apical side (Brown et al., 2008). Therefore, it is puzzling that cell types like hRBC, which have an active uptake of creatinine, lacks P-glycoprotein, and MRP2 (Kock et al., 2007).

Human OAT2 transports/interacts with creatinine (K_m-value of 795 μM) in human OAT2-transfected HEK cell line (Shen et al., 2015). The present study of hRBC shows virtually identical K_m-value (854 μM) for creatinine uptake in hRBC. In both studies typical Michaelis–Mentens curves were observed. As far as we know, OCT2 has not been identified in hRBC cell membranes. The co-incubation of creatinine and cyclic nucleotides gave an unexpected observation. Creatinine caused reduced uptake of cyclic nucleotides, but had the characteristics of uncompetitive inhibition. In classical enzyme kinetics uncompetitive inhibition is characterised with increased affinity (K_m ↓) and lowered maximal transport capacity (V_{max} ↓). An uncompetitive inhibitor only recognises and interacts with the transporter when the substrate is bound. In addition, inhibition increases with higher substrate concentrations. Such a mechanism may protect cells against high basal cyclic nucleotide levels. Uncompetitive inhibition may be an additional property that distinguishes hOAT2 from other members of the OAT-family. The transporter has been characterised as an uniporter (Fork et al., 2011) and cGMP was not transported via exchange of monocarboxylates, dicarboxylates, or hydroxyl ions (Henjakovic et al., 2015). The driving force for cyclic nucleotide uptake has not been identified.

The present study showed an uptake of indomethacin to intact cells with a K_m-value of ≈500 μM). The present study showed that cyclic nucleotides interacted with the indomethacin in a competitive manner. In OAT2-transfected HEK-293-tv1 cells, indomethacin (100 μM) inhibited cGMP uptake by ≈ 95% (Henjakovic et al., 2015), and ≈75% (Shen et al., 2015). The IC_{50}-values for indomethacin was 2.1 μM for the interaction with creatinine (Shen et al., 2015) and 3.7 μM for the interaction with penciclovir (Hotchkiss et al., 2015). The markedly lower affinity observed for intact hRBC may have its origin in a simultaneous uptake and efflux of indomethacin, being a competitive MRP4 substrate (Reid et al., 2003).

PAH inhibited accumulation of cGMP in a concentration-dependent manner in kidney cortex from rats (Gemba, Kawaguchi, Konishi, Nakanishi, & Matsushima, 1983). Based on this report we included PAH to study the effect on cyclic nucleotide hRBC uptake. A temperature-sensitive and saturable PAH uptake existed but with markedly lower affinity (2,000 μM) than reported for human OAT1 (K_m ≈ 28.5 μM (range 2.1–113 μM) (Burckhardt, 2012). Again, the intact cell model may have caused an underestimation of affinity. The PAH uptake is paralleled with efflux by MRP4 with relative high affinity (K_m ≈ 160 μM) (Smeets, van Aubel, Wouterse, Van Den Heuvel, & Russel, 2004). Based on the same iterative approach as for cyclic nucleotides a real K_m-value of 100–150 μM could exist. The V_{max} for PAH was approximately 0.1% of that observed for cyclic nucleotides and indomethacin.

PAH has been the typical test substrate for the OAT1 expression in stably transfected cells (Henjakovic et al., 2015), but also used in knock-out models. The renal clearance of PAH was markedly diminished (≈75%) in renal slices from OAT1 knock-out mice in comparison with slices from wild-type animals (Eraly et al., 2006). In another study, knockout of OAT3 reduced PAH renal clearance but less than in OAT1 knockout mice (Sweeney et al., 2011). A close correlation between the mRNA expression of hOAT1 and hOAT3 was found in human kidney slices (Nozaki et al., 2007) and the correlation of saturable PAH uptake suggest a role of OAT3 in the transport of PAH. In our hands Western blotting did not reveal OAT1 or OAT3 in hRBC cell membranes. In agreement, PAH showed no competition with uptake of indomethacin, cGMP, or cAMP. Earlier studies claimed that PAH was a hOAT2 substrate but a recent review questions this (Shen, Lai, & Rodrigues, 2017).

The present study shows expression of OAT2 in hRBC and that the transporter is functionally active with uptake of organic anions like cyclic nucleotides, creatinine, and indomethacin. The present results argue against PAH as a substrate for hOAT2.

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