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Original scientific paper

Fluorescent organic cations for human OCT2 transporters screening: uptake in CHO cells stably expressing hOCT2

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Abstract

The aim of this study was to assess the suitability of amiloride, rhodamine 6G and rhodamine 123 as non-radioactive substrates for characterizing hOCT2 using CHO cells. The uptake characteristics of these compounds were compared in wild-type (WT) and human organic cation transporter 2 (hOCT2)-stably transfected Chinese Hamster Ovary (CHO) cells. All the compounds were accumulated by the CHO-hOCT2 cells. Intracellular uptake of the compounds was higher in CHO cells stably-expressing hOCT2 compared to the WT. The uptake was concentration–dependent and saturable (except for rhodamine 123). The affinities of the compounds for the hOCT2 (in descending order) were: amiloride (K_m = 72.63 ± 12.02 µM) > rhodamine 6 G (K_m = 82.47 ± 29.15 µM). Uptake of amiloride in transfected cells was pH -dependent and significantly inhibited by hOCT2 inhibitors (quinine, verapamil and quinidine). Based on our kinetic data and other considerations, we recommend the use of amiloride for characterizing hOCT2 transporters.

Keywords

Transporters; OCT2; CHO cells; amiloride; rhodamine; fluorescence

Introduction

Organic cations are positively charged amines or organic molecules with transient or permanent net positive charge(s) at physiological pH [1]. A wide variety of clinically used drugs (e.g. cimetidine, procainamide, quinidine, salbutamol) and endogenous bioactive amines (e.g. dopamine, histamine, N-methylnicotinamide, choline) are organic cations [1-2]. These charged molecules are transported across cell epithelia by carrier-mediated transport proteins, mainly organic cation transporters (OCTs) and proton/cation antiporters (MATEs) [3-6]. These transporters play pivotal roles in the absorption, distribution and disposition of cationic drugs in various tissues. The OCTs are members of the solute carrier family, SLC22A [3-4, 7]. The SLC22 family is sub-grouped according to substrates and based on the driving force for the cation transport mechanisms [1, 3-4]. One of such subgroups, which translocate organic

cations and weak bases in an electrogenic-, sodium chloride- and proton gradient-independent manner, is the SLC22A1-3 (OCT1-3). These facilitative diffusion systems transport organic cations in both directions across the plasma membrane. Other SLC22 transporter subgroups are the sodium- and pH-dependent cation/carnitine transporters, SLC22A4 (OCTN1) and SLC22A5 (OCTN2) [8-10]. Functionally, the OCTs and OCTNs play a crucial role in the regulation of cellular functions. Their involvement in cationic solute absorption, transport and excretion in the intestinal, placental, nasal and renal epithelia is well documented [10-11].

Among the organic cation transporters, the OCT2 is the transporter with the potential to significantly alter drug disposition. Based on this fact, the US Food and Drugs Administration agency (FDA) and European Medicines Agency (EMA) require that new drug candidates be screened for OCT2 activity during early stages of drug development if active renal excretion is envisaged [12-13]. The transporter is mainly expressed in the basolateral membrane of renal proximal tubules and is also expressed in small intestine, lung, placenta, thymus, brain and the inner ear [2, 14-15]. Although the role of the transporter in other tissues than the kidney is still under investigation, the OCT2 is critical for the detoxification and elimination of drugs and endogenous compounds from the systemic circulation and thus a significant determinant of drug response and sensitivity [2, 4].

The most commonly used method for membrane transporter characterization is radioactivity. However, this approach is neither convenient nor cheap due isotope handling, disposal measures and regulatory restrictions. Furthermore, there is potential health risk associated with the use of radiolabelled substrates if handled incorrectly. Currently, there is a growing interest in the development of low-risk screening technologies for drug transporters characterization [16-17]. Unlike radioactivity, fluorescent assays significantly improve laboratory safety with the opportunity for real-time monitoring using fluorimeters. Currently, 4-Di-1-ASP is the main fluorescent probe for screening organic cation transporters. It has a permanent positive charge, high fluorescent intensity and stable over a range of pH and temperature. However, the compound is transported by all OCT isoforms (OCT1-2, OCTN1 and OCTN2). It is not among the compounds recommended by The International Transporter Consortium for investigating OCT2 [18]. It is therefore relevant to explore the use of other fluorescent organic cation probes that may specifically interact with OCT2. The objective of this study was to investigate the potential use of amiloride, rhodamine 123 and rhodamine 6G as specific fluorescent substrates for hOCT2 using CHO cells stably expressing the transporter. To the best of our knowledge, there is no published study on potential interaction between hOCT2 stably expressed in CHO cells and the fluorescent substrates described in this study.

Experimental

Chemicals

Recovery™ Cell Culture Freezing Medium, Kaighns F12, 0.25% Trypsin-EDTA, phenol red, Zeocin™ Selection Reagent, Hygromycin B, DMEM-F12, Salmon sperm DNA, Fetal bovine serum and phosphate-buffered-saline (PBS) were supplied by Life Technologies (Burlington, ON Canada). Triton X-100(R), verapamil, quinine, quinidine, corticosterone, bovine serum albumin (BSA), isopropanol, Hanks' balanced salt (HBSS), and penicillin/streptomycin were purchased from Sigma (St. Louis, MO, USA). Rhodamine 123, rhodamine 6G and amiloride were procured from Santa Cruz Biotech Inc. (Dallas, Texas, USA). Bicinchoninic acid (BCA) protein assay kit was from Millipore (Billerica, MA, USA) for protein assay according to the method described by Smith et al [19].

Chinese Hamster Ovary (CHO) Cell Culture and Stable Expression of hOCT2

CHO cell was chosen for stable expression of hOCT2 as it is one of the models / cultured cell lines recommended by The International Transporter Consortium for studying drug transporter interactions so as to assess uptake and or efflux by single recombinant transporters [18]; and there is no published study on potential interaction between hOCT2 stably expressed in CHO cells and the fluorescent substrates .The CHO cells used were from ATCC (Manassas, VA, USA).

For stable expression of hOCT2, cells were electroporated with 10 µg of hOCT2-pcDNA3.1 plasmid DNA and 10 µg of salmon sperm DNA in a cuvette (4-mm gap) using a BTX ECM 630 electroporator (Harvard Apparatus, St. Laurent, Quebec, Canada) with settings of 1050 microfarad, 260 V, and at no resistance. Forty -eight hours after the electroporation, positively transfected cells were selected with 200 µg/ml Hygromycin B. The CHO wild type were not transfected with hOCT2 as the cells will be used as the control. The CHO cells were grown in Ham's F-12 (Kaighn's Modification) containing 10 % FBS, 1 % penicillin-streptomycin, 200 µg/ ml Hygromicin B (transfected) and 100 µg /ml Zeocin (wild-type), respectively. Both cell types were maintained in a humidified atmosphere (5 % CO_2/95 % O_2). The cells were passaged every 2-3 days. Cells within passage 4–35 were used for the studies.

Uptake studies

Confluent CHO-hOCT2 and wild –type cells cultured in 24- , 48- or 96 well plates were used for uptake experiments. The uptake buffer was prepared with HBSS supplemented with 25 mM glucose and 20 mM HEPES (pH 7.4). The cells in each well were pre-incubated in substrate-free uptake buffer (200 µl) at 37 °C for 15 min. Time-dependent studies were conducted by incubating cell monolayers with 10 µM cationic compounds for up to 60 min at 37 °C. Uptake studies were initiated by replacing the pre-incubation substrate-free buffer with buffer solutions containing 200 µl of rhodamine 123, rhodamine 6G, and amiloride, respectively. Based on preliminary studies, the experiments were terminated at 1, 2, 5, 15, 30 and 60 min by immediately removing the compounds and washing the cells three times with ice-cold HBSS. Subsequently, the cells were dissolved in 200 µl of 1% Triton X-100R containing 0.1 M NaOH on a shaker for 1–2 hours at 37 °C. Furthermore, uptake of the fluorescent substrates was investigated at different concentrations (1-3000µM) and pH values (5.0-8.5). In studies involving inhibition, the cells were pre-incubated at 37 °C with or without inhibitors for 15 min prior to the uptake studies. Each set of experiments was performed with cells of the same age and passage.

Sample analyses

Rhodamine-123, rhodamine-6G, and amiloride in samples were quantified with Modulus single tube multimode florescence reader (Fluorimeter model 9200, Turner Bio systems, Sunnyvale, CA, USA) using the green, blue and UV filter configurations for rhodamines, and amiloride, respectively. Protein content of cell lysates was measured using bovine serum albumin as standards according to BCA protein assay protocol so as to normalize the cell population/density in each plate [19].

Data analyses.

The saturable uptake of the fluorescent substrates was analyzed by assuming Michaelis-Menten type carrier-mediated transport. The maximum transport rate (Vmax), the Michaelis constant (K_m), and IC50 for the inhibition studies were determined by non-linear regression using Prism(R) 5.0 (GraphPad, San Diego, CA, USA). Passive diffusion was accounted for by subtracting the rate constant for the compounds passive diffusion in CHO-wild cells from CHO-hOCT2-expressing cells. Unless stated otherwise, all experiments

were performed in triplicates and data presented as mean ± SD. Where appropriate, statistical significance of the results was determined with ANOVA using InStat® 3.0 (GraphPad, San Diego, CA, USA). $P < 0.05$ was considered significant. The specific uptake of the dyes was calculated by subtracting the unspecific uptake in Wild CHO cells from the total uptake in CHO-hOCT2 cells.

Results and Discussion

Considering the fluorescence characteristics of rhodamine-123 and rhodamine-6G (high quantum yield, long emission and excitation wavelengths) their extensive current biomedical applications, and the fact that these compounds are organic cations [17], we selected them for this study. Furthermore, Rhodamine 123 was used based on the fact that it is an organic cation transporter used for characterizing OCT2 [20] Amiloride was chosen because it is the only OCT2 substrate with desirable fluorescent characteristics listed by the International Transporters Consortium for OCT2 transporter characterization [18]. The hOCT2 is a very important OCT isoform that exhibits clinically relevant genetic polymorphisms, participates in clinical drug–drug interactions and alters the disposition and excretion of some drugs [18].

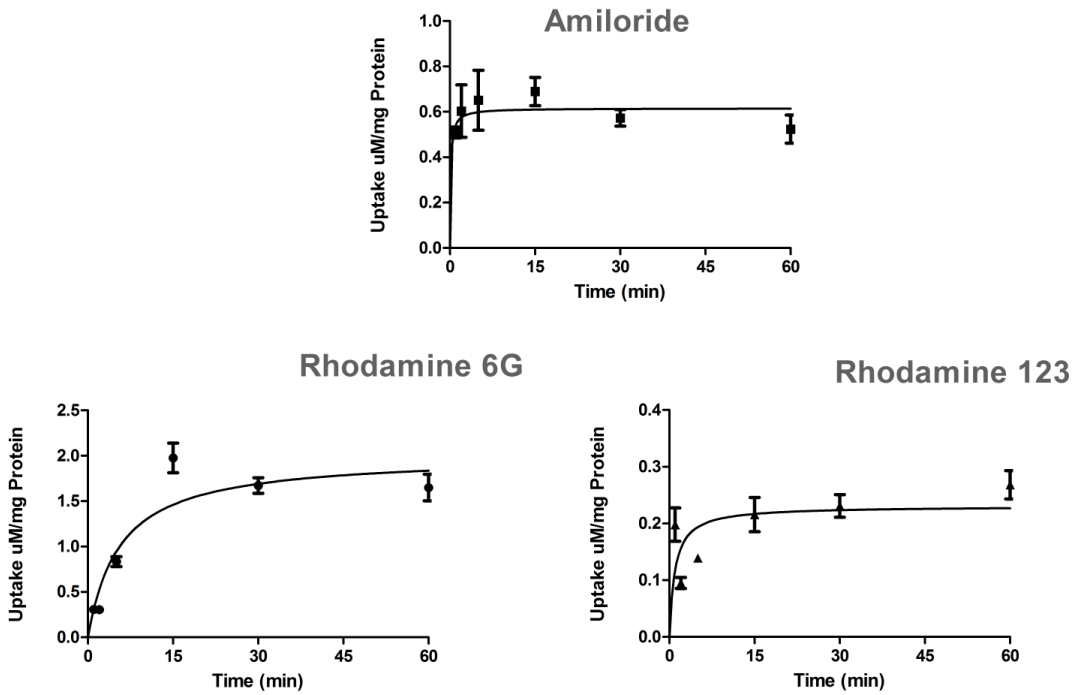

Figure 1. Time-dependent uptake of the fluorescent compounds into CHO-hOCT2. Cell layers were incubated with (10 μM) of each compound as indicated. Subsequently, the cells were washed with ice-cold uptake buffer followed by extraction with Triton X-100/0.1 M NaOH. Data represent mean ± SD; n = 3.

Figure 1 shows the time–dependent uptake of the substrates by CHO cells stably expressing hOCT2. Uptake of the three compounds was linear up to approximately 7 min; after which a gradual plateau was observed. Based on these observations, an incubation period of 5 min was chosen for subsequent experiments. The effect of extracellular pH on the uptake of amiloride, rhodamine 6G and rhodamine 123 by CHO-hOCT2 and CHO-WT cells is summarized in Figure 2. The three compounds accumulated in the cells in a pH-dependent manner. Highest uptake was observed at alkaline pH range of 8.0 - 8.5 for CHO cells stably-expressing hOCT2. Conversely, decreased uptake was seen in acidic pH range. For CHO-WT cells, no clear pattern of pH-dependency was deciphered. The observed pH effect suggests that pH-sensitive organic cation transporters may have contributed to the observed solute transport as no clear pH-dependent

pattern was seen in CHO-WT cells. The pH effect for amiloride was comparable and in agreement with published data [9,17,21]. These studies involving the uptake of 4-Di-1-ASP by OCTs in Calu-3, A549, 16HBE14o- and Caco-2 cells demonstrated higher substrate accumulation under alkaline extracellular pH range.

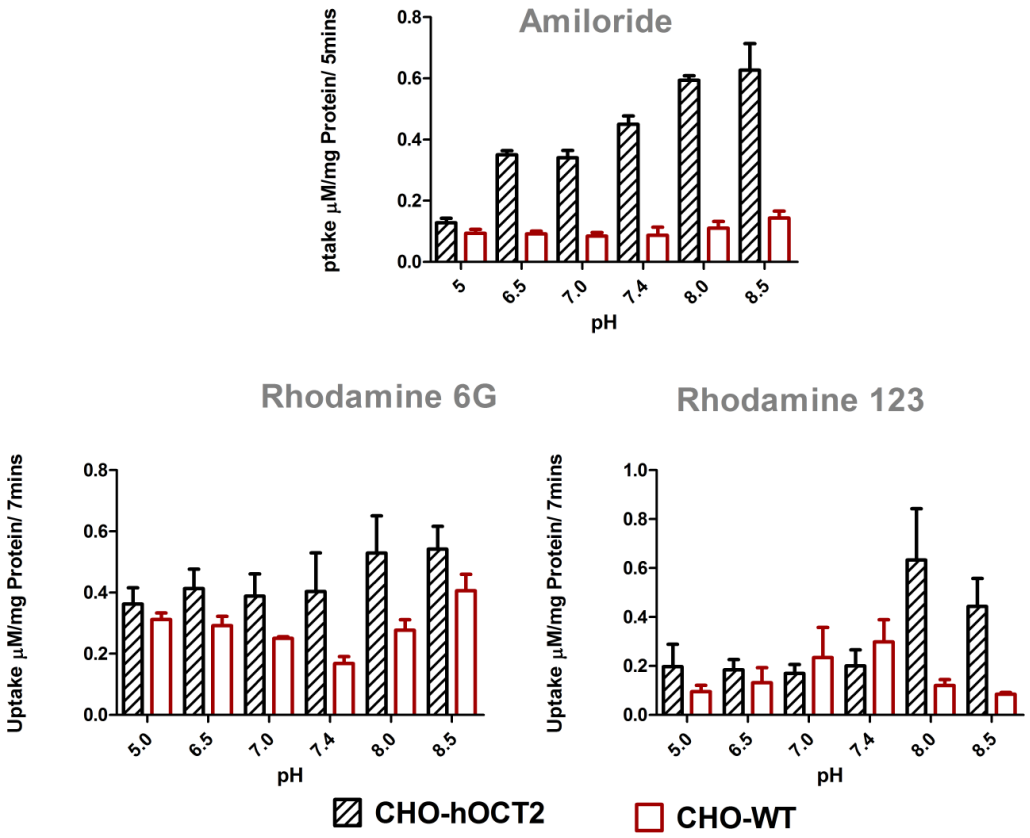

Figure 2. Effect of extracellular pH on uptake in CHO cells stably expressing hOCT2 and CHO wild type. Uptake of the compounds (10µM) was evaluated at 37 °C for 7 min using CHO-hOCT2, and CHO-WT cells. Subsequently, the cells were washed with ice-cold uptake buffer followed by extraction with Triton X-100/0.1 M NaOH. Data represent mean ± SD; n = 3.

If the uptake of the fluorescent cations was mediated by hOCT2, concentration-dependent and saturable uptake would be expected. Figure 3 shows the effect of concentration on amiloride, rhodamine 123, and rhodamine 6G uptake by CHO-hOCT2 and CHO-WT cells. A clear concentration–dependent effect was observed for CHO-hOCT2 cells. At various concentrations, amiloride, rhodamine 6G and rhodamine 123 were accumulated to a greater amount by CHO-hOCT2 cells compared to CHO-WT cells. Uptake of the compounds in CHO-hOCT2 cells (except rhodamine 123) was saturable. The calculated kinetic parameters are: K_m = 72.63±12.02 µM, V_{max} = 0.027±0.001 µM/mg protein/min (amiloride) and K_m = 82.47±29.15 µM, V_{max}= 0.019±0.002 µM/mg protein/min (rhodamine 6G), respectively. We were unable to determine the kinetic parameters for rhodamine 123 due to excessive non-specific binding to the 96-well plates at higher concentrations. These results indicate that amiloride and rhodamine 6G appear to be better substrates for hOCT2 compared to rhodamine 123. The rank order of affinity of the dyes based on Michaelis-Menten modelling is: amiloride (K_m = 72.63 ± 12.02 µM) > rhodamine 6G (K_m = 82.47 ± 29.15 µM) > rhodamine 123 (K_m = ND). As Km is a measure of transporter-substrate affinity [22-23], higher K_m implies poorer binding/affinity. Biermann et al., [2] used Km to compare the affinities of different fluorescent compounds to hOCT2 in transfected HEK-293. According to the authors, amiloride and 4-Di-1-ASP had significantly higher uptake rates in hOCT2 cells compared to wild type cells. The km obtained in this study was

comparable to the value (95 µm) obtained by Biermann et al using HEK-293 stably expressing hOCT2. Amiloride is not only taken up by cells, but can be transported. This behaviour may be significantly affected by the degree of ionization of the compound. The guanidine moiety of the compound is protonated at physiologic pH, and it is this positively charged species that makes it an organic cation [24]. As a weak base, the degree of ionization plays a critical role in its binding to organic cation transporters [25]. This implies that permeation at pH much lower than the p*K*a of the compound may be by passive diffusion. For the cationic rhodamines investigated, there was no clear-cut pH effect within the range investigated. This was not surprising because we previously showed that fluorescence intensity of rhodamine-6G is characteristically acid-dependent due to molecular changes and transitions [17]. Under our experimental conditions, it was not possible to differentiate the effect of this phenomenon on uptake from the impact of pH changes on uptake.

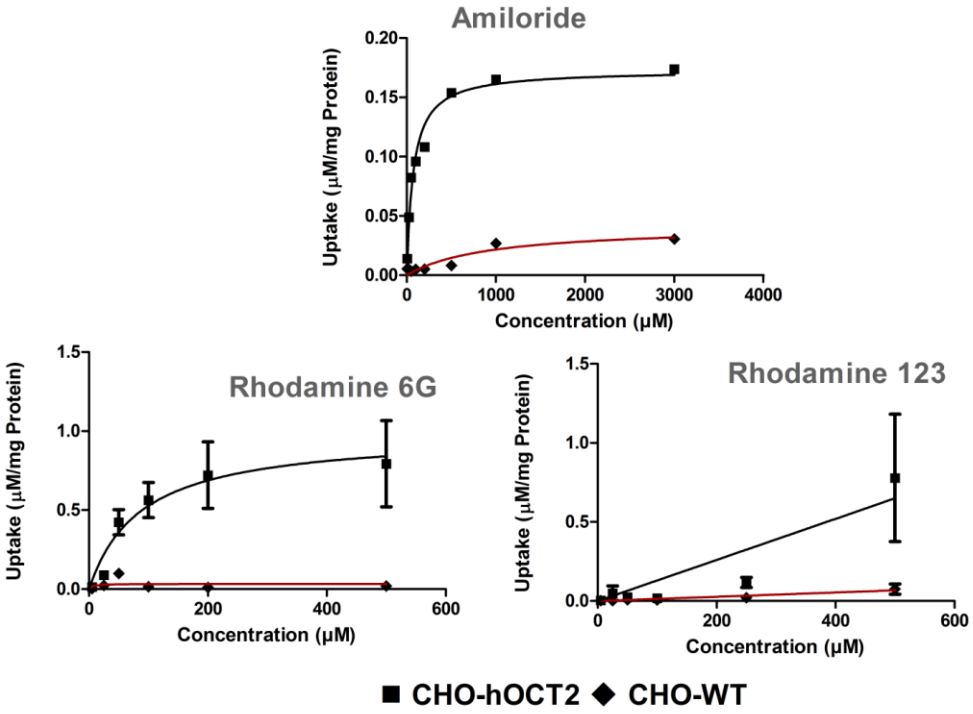

Figure 3. Concentration-dependent intracellular uptake of the fluorescent compounds in CHO-hOCT2 and CHO-WT. Uptake was evaluated at 37 °C for 7 min. Subsequently, the cells were washed with ice-cold uptake buffer followed by extraction with Triton X-100/0.1 M NaOH. Data represent mean ± SD; n = 3.

If the uptake of the cationic fluorochromes were mediated by hOCT2, one would expect hOCT2 inhibitors to differentially reduce the uptake of these compounds in transfected cells (Figures 4-7). We used six inhibitors (Figure 4) at concentration of 1mM for initial screening after which the three most potent (quinine, verapamil and quinidine HCl) were selected for a concentration dependent inhibition studies (Figure 5). These inhibitors have been reported by other published literature as potent and OCT specific inhibitors [9, 10, 17]. Figure 4 shows the effect of hOCT2 and OCTN1-2 inhibitors and substrates on amiloride, rhodamine 6G and rhodamine 123 uptake in CHO-hOCT2 cells. At 1mM, quinidine, quinine and verapamil consistently inhibited amiloride, and rhodamine 6G uptake. Expectedly, OCTN inhibitors (NMN, choline and guanidine) had no significant effect on the uptake of these compounds ($p>0.5$), an indication that their uptake was likely due to hOCT2 transporters. To confirm the affinity data obtained with Michaelis-Menten kinetics, concentration-dependent inhibition of amiloride, rhodamine 6G, and rhodamine 123 using hOCT2 inhibitors (quinine, quinidine, and verapamil) were investigated (Figures 5-7). Figure 5 shows the effect of increasing concentrations of quinidine on the fluorescent compounds uptake.

The IC50s for the inhibition were 891 μM (amiloride), 315 μM (rhodamine 123) and 1137 μM (rhodamine 6G). Similarly, Figure 6 summarizes concentration-dependent inhibition of the cationic dyes by quinine. The IC50s for the inhibition were 309.6, 279, and 206 μM for amiloride, rhodamine 123 and rhodamine 6G, respectively. Data for inhibition studies with verapamil is shown in Figure 7. Verapamil concentration-dependently inhibited amiloride (IC50 = 486 μM), rhodamine 123 (IC50 = 302 μM) and rhodamine 6G (IC50 = 583 μM). Inhibition of the cationic compounds by quinine, quinidine and verapamil, suggests that these compounds may share the same substrate recognition sites. Previous reports from our laboratory and others have shown an interaction between quinine, verapamil, quinidine and organic cation transporters [2, 9, 10, 17]. Quinine blocked Et+ uptake by cells overexpressing hOCT1 or hOCT2 with high affinity [26]. Similarly, quinine and verapamil inhibited hOCT1 and hOCT2-mediated uptake of rhodamine 123 with higher inhibitory effects observed for hOCT2 [20]. The apparent discrepancy in the IC50 values could be attributed to the fact that amiloride, and rhodamines bind to different, but partially overlapping sites in the binding pockets of the hOCT2. The hOCT2 was previously reported to be polyspecific, as such can accept compounds of different sizes and molecular shapes [2, 4, 27]. Thus it exhibits large variations in affinity and turnover for different compounds. The observed IC50 values for quinine and verapamil varied with the reported IC50 of 23 μM of quinine [28] and 85 μM of verapamil [29] in HEK 293 cells expressing OCT 2 using N-methylpyridinium as substrate. Similarly the observed IC50 of quinidine is different from 13.3 μM in a CHO cell expressing OCT2 using N-methylpyridinium as substrate [30]. The presence of various substrate and inhibitor binding sites and the complex interactions between different sites explains why largely different IC50 values are obtained for individual transporters when different substrates are used for transport measurements [2,4]. Volk et al. [31] showed that the substrate binding site of rOCT2 was complex, and it exposes different interactive domains for different substrates and inhibitors. A similar analogy could be true for hOCT2 because human OCT2 show about 90 % amino acid identities to the rat OCT2 [32]. Several of these amino acids in the transporter transmembrane helix contain high- and low-affinity substrate and/or inhibitor binding sites that are involved in substrate and/or inhibitor binding of the OCT transporters [4,22]. In line with the conformational analogy, Thévenod and co-workers maintained that OCTs acquire minimum of three conformational states: one state with an outward-open binding cleft containing an innermost binding pocket where transported cations bind and additional more peripherally localized binding sites for organic cations which may overlap with domains in the innermost binding pocket [31]. They reported that structurally different transported cations induce substrates due to the interaction of different substrates with partially different amino acids [31]. In addition to overlapping sites, they also reported that the inhibitor potencies of OCT ligands are highly dependent on the substrate employed for uptake studies.

The uptake of the fluorescent compounds (amiloride, rhodamine 6G and rhodamine 123) was OCT2-mediated. This was evident in the concentration- dependent uptake of the compounds, as well as their uptake inhibition by classical organic cationic inhibitors.

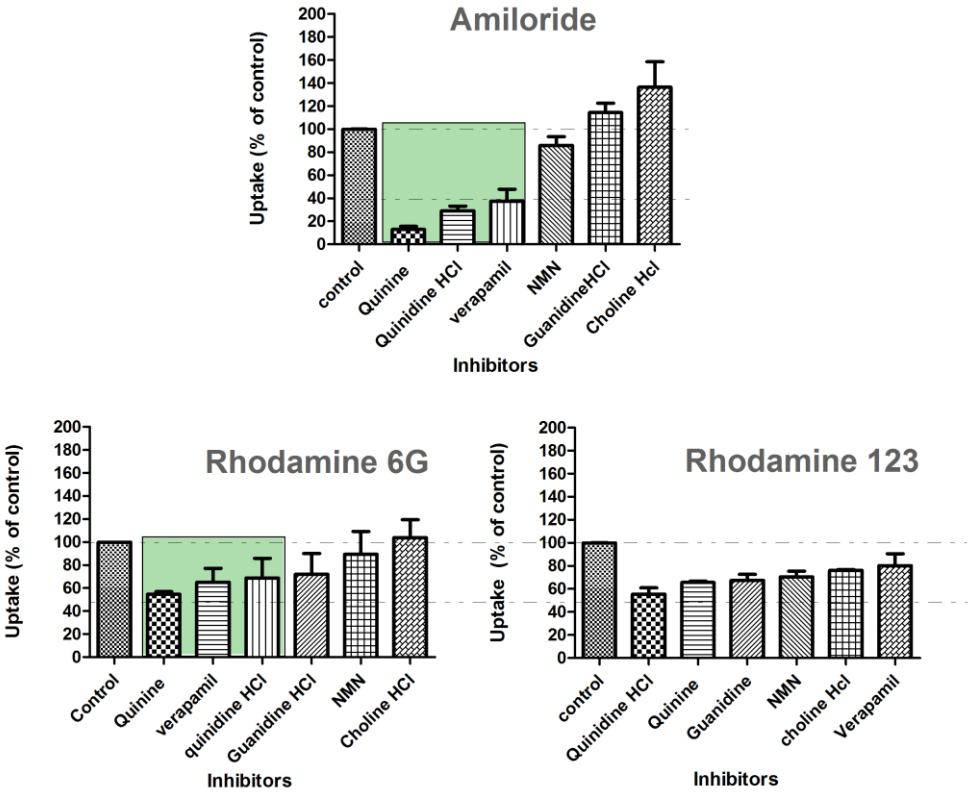

Figure 4. Inhibition of the fluorescent compounds uptake in CHO cells stably expressing hOCT2 by OCT/OCTN inhibitors. Cells were pre-incubated with inhibitors at 1 mM for 25 min followed by an additional 7 min incubation with the respective fluorescent compounds. Subsequently, the cells were washed with ice-cold uptake buffer followed by extraction with Triton X-100/0.1 M NaOH. Data represent mean ± SD; n = 3.

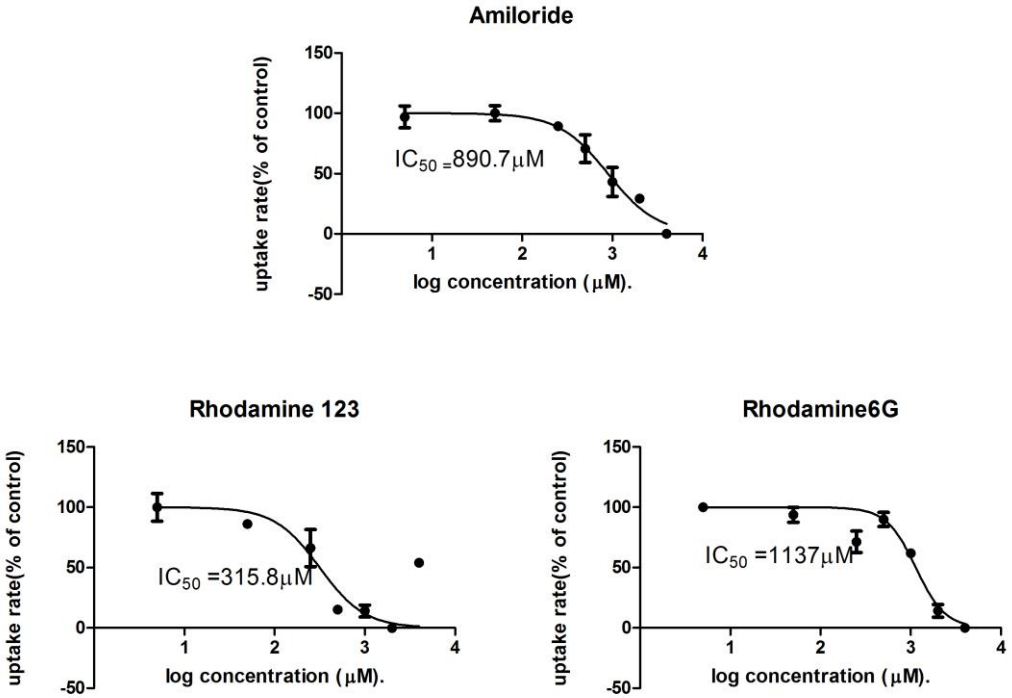

Figure 5. Inhibition of the fluorescent compounds uptake in CHO cells stably expressing hOCT2 by Quinidine. Cells were pre-incubated with inhibitor (Quinidine) for 25 min followed by additional 7 min incubation with the respective dyes. Subsequently, the cells were washed with ice-cold uptake buffer followed by extraction with Triton X-100/0.1 M NaOH. Data represent mean ± SD; n = 3.

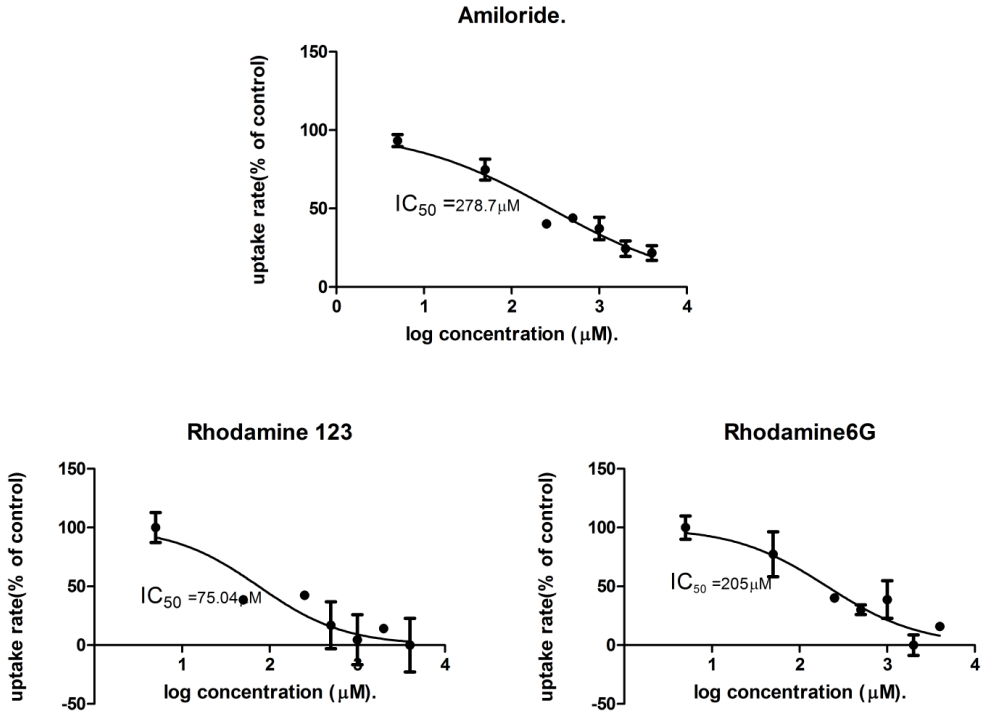

Figure 6. Inhibition of the fluorescent compounds uptake in CHO cells stably expressing hOCT2 by Quinine. Cells were pre-incubated with inhibitor (Quinine) for 25 min followed by an additional 7 min incubation with the respective dyes. Subsequently, the cells were washed with ice-cold uptake buffer followed by extraction with Triton X-100/0.1 M NaOH. Data represent mean ± SD; n = 3.

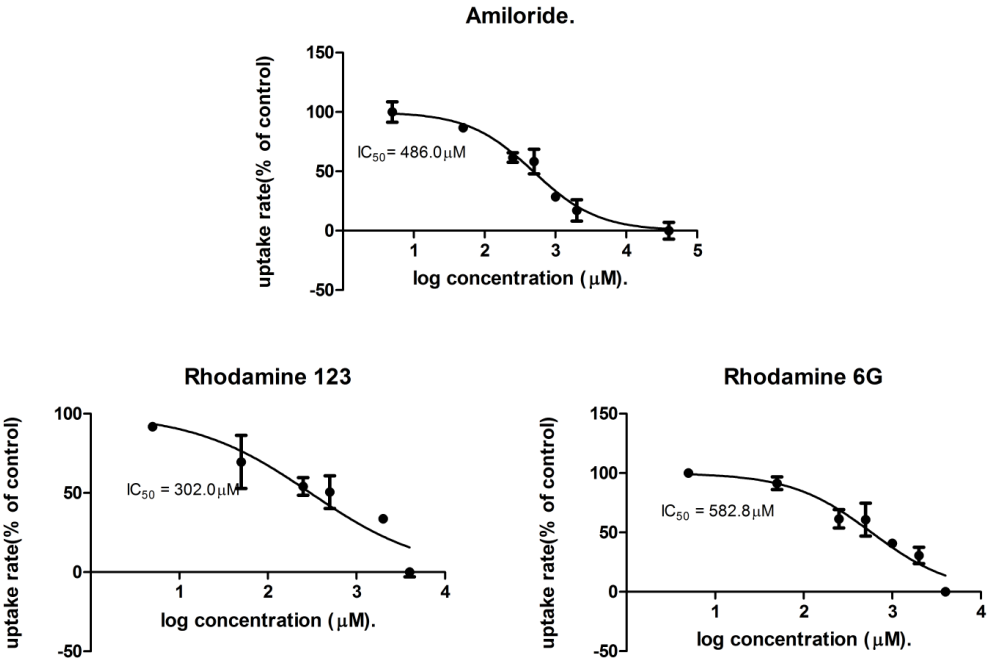

Figure 7. Inhibition of the fluorescent compounds uptake in CHO cells stably expressing hOCT2 by Verapamil. Cells were pre-incubated with inhibitor (Verapamil) for 25 min followed by additional 7 min incubation with the respective dyes. Subsequently, the cells were washed with ice-cold uptake buffer followed by extraction with Triton X-100/0.1 M NaOH. Data represent mean ± SD; n = 3.

Conclusions

In this study we investigated the potential use of amiloride, rhodamine 6G and rhodamine 123 as non-radioactive probes for characterizing human organic cation transporter isoform, hOCT2 using CHO cells stably-expressing the transporter. Our data suggest that the investigated fluorescent organic cations were efficiently taken up by the CHO cells. However, based on their fluorescence, affinities (K_m/IC50s), aqueous solubility, ease of use at high concentrations and recognition as hOCT2 substrates by FDA and European Union and the possibility to be used for in vivo human studies, we recommend the use of amiloride as an acceptable non-radioactive substrate for screening hOCT2 expression in cells using fluorimeters. Although amiloride is a substrate for organic cation transporters, it also inhibits sodium reabsorption in the kidney via the blockade of Na^+/H^+ antiporter located on the apical side of the proximal tubules. From drug interaction perspective, care should be taken in geriatrics or patients with hyperkalaemia or those receiving other potassium-sparing agents when using this drug. Furthermore, considering the mechanism of action of amiloride and the role and wide expression of OCT2 in kidney, the use of this drug along with clinically used potent OCT2 inhibitors (e.g. metformin), should be used with caution to avoid clinically significant interactions – in this case hypoglycaemic (reduced metformin clearance) or elevated amiloride concentration and duration (due to metformin inhibition), which may be an advantage considering amiloride's short duration of action. It is also important to note that OCT2 transporters are mainly expressed basolaterally in the kidney tissues, which makes individuals with polymorphisms of this transporter prone to clinically significant drug toxicity due to reduced renal clearance. Overall, observed clinical manifestation of genetic polymorphisms involving amiloride may depend on whether the genes involved affects Na+/H+ antiporter located apically or OCT2 located basolaterally.

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