Lack of interaction of the fluorosurfactant C6O4 with human renal transporters: In vitro/*in silico* analysis

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ABSTRACT

C6O4 is a water soluble perfluoroether carboxylic acid ammonium salt used as surfactant in the synthesis of fluoropolymers. Available experimental data in rats exposed by the oral route indicate it is eliminated in urine. Previous studies with various linear perfluorocarboxylic acids have suggested that these compounds are substrates of renal membrane transporters in rats and humans, and that the interaction with basal and apical membrane transporters can influence the elimination kinetic by these organisms and explain, in part, the observed differences in the respective half-lives. In particular, apical transporters may contribute to the reuptake of these exogenous compounds from the tubule lumen. The present study was designed to investigate the uptake of C6O4 in two renal cell lines transiently transfected with the human apical membrane transporters, organic anion transporter 4 (OAT4), and urate transporter 1 (URAT1). The uptake of the linear perfluorohexanoic acid (PFC6) was evaluated in parallel. While the uptake of the conjugated steroid estrone-3-sulfate (E3S), a known substrate for renal transporters, and of PFC6 was clearly observed in both cell types transfected with either OAT4 or URAT1, no significant uptake of C6O4 was measured under the same test conditions. The results of the transporter's functionality measured in vitro were consistent with molecular docking simulations. Both outward and inward models of the transporters showed a reduced interaction between C6O4 and URAT1 or OAT4. In contrast, more stable interactions were predicted for PFC6 and PFOA, as well as for the E3S substrate, as shown by the respective docking scores reflecting the binding strength and by the poses assumed in the transporter channels. Altogether, the in vitro and *in silico* modeling results showed a low reuptake potential and limited interactions of C6O4 molecule with two human apical membrane transporters, contrasting with the more efficient reuptake of PFC6 from the tubule lumen. These results suggest reabsorption from the proximal tubule by apical renal transporters is not likely to interfere with the elimination pathway of C6O4 in humans.

1. Introduction

Perfluorocarboxylic acids (PFCAs) are used in a variety of industrial applications and in the manufacture of consumer products. A number of PFCAs, including perfluorooctanoic acid (PFOA), have been detected in the environment and in the human population, with concerns that their potential accumulation and persistence in the human body for long periods of time could lead to adverse health effects (Conder et al., 2008; Fenton et al., 2021; Vestergren and Cousins, 2009).

PFOA is excreted primarily via the kidney and its biopersistence, found to be sex- and species-dependent, may be a function of renal proximal tubular reabsorption, a process regulated by the interaction of the molecule with organic anion transporter (OATs) proteins located on the tubular epithelial cell membranes (Han et al., 2012). These transport

proteins are part of the major SLC22 transporter family. In the kidney, these proteins are present in the apical and basolateral membranes of proximal tubule cells and play an important role in the excretion and tubular reabsorption of endogenous metabolites as well as xenobiotics (Burckhardt, 2012; Nigam et al., 2015; Otani et al., 2017; Zou et al., 2021). In humans, the most abundant renal transporter proteins are those encoded by the genes OAT1 (SLC22A6), OAT3 (SLC22A8), OCT2 (SLC22A2), and ORCTL2 (SLC22A18) (Oswald et al., 2019), and are localized on the basolateral membrane of the cells.

Yang et al. (Yang et al., 2010), demonstrated that proteins encoded by OAT4 (SLC22A11) and URAT1 (SLC22A12) genes are key transporters present in the apical membrane of the proximal tubule and are involved in the renal reabsorption of PFCAs in humans. PFCAs are also capable of inhibiting the activity of these transporters, with a level of inhibition dependent on the compound chain length (C7 >C8 >C9 >C10), resulting in reabsorption of the molecule and inhibition of excretion (Yang et al., 2010). OAT4 has also been reported to facilitate reabsorption of conjugated steroids, such as E3S with a high affinity (Cha et al., 2000; Nakagawa et al., 2009). The interaction of PFCAs with urate reuptake transporters could contribute to their reabsorption and explain their long persistence in the organism.

C6O4 is a perfluoroether carboxylic acid, ammonium salt, used as polymerization aid in the synthesis of fluoropolymers and developed as a substitute to the legacy PFCAs. Available data in rats indicate C6O4 is distributed systemically and quite rapidly following oral ingestion, causes liver effects but has a short half-life in blood and its main elimination pathway is urine, similarly to other PFCAs ("Registration Dossier - ECHA, ", 2022). However, there is currently no reported data regarding human exposure to this compound and its half-life in humans.

Here, to get some insight on the renal elimination mechanisms in humans, we aimed to determine whether C6O4 is a substrate for OAT4 and URAT1 transporters, through an integrated in vitro/*in silico* approach that includes the use of transfected human renal cells and docking simulations.

2. Material and methods

2.1. Chemicals

C6O4 (CAS RN 1190931–27–1, purity: $\geq$ 97.5%), also known as cyclic C6O4 (cC6O4), was provided by Solvay Specialty Polymers Italy S.p.A., perfluorohexanoic acid (PFC6, CAS 307–24–4, purity: $\geq$ 97.0%), estrone-3-sulfate (E3S, CAS 1240–04–6), acetonitrile (HPLC grade) and Ammonium Acetate (AcONH$_4$) were purchased from Merck Life Science S.r.l., and 5-bromo-2′-deoxyuridine (BrdU) was purchased from Roche Applied Science.

2.2. Cell cultures and transfection

Two human renal tubular cell lines were used for functional assays. HK2 cell line, purchased from ATCC (LGC Standards S.r.l.) was cultured in Keratinocyte Serum Free Medium (K-SFM, Invitrogen, Life Technology). HEK293 cell line, purchased from Sigma-Aldrich (Merck Life Science S.r.l.) was cultured in EMEM with 2 nM L-Glutamine (Lonza), antibiotics (100 U/ml of Penicillin/Streptomycin, Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS).

HK2 and HEK293 cell lines were transfected with the pCR3.1 expression vector containing the human OAT4 and URAT1 genes coding sequence (Supplementary Table S1) or with the empty plasmid (pCR3.1, Life Technologies), or with the same pCR3.1 expression vector containing the green fluorescent protein (GFP) gene as a control of transfection. Briefly, cells growing in 6-well culture plates were treated with Lipofectamine LTX & Plus Reagent (Life Technology) and 2.5 μg/well of OAT4- and URAT1-containing plasmid or the same amount of empty pCR3.1 or of GFP-containing plasmid.

2.3. Analysis of cell proliferation

Renal tubular cells were cultured in 96-well plates (5,000 cells/well) in complete medium for 24 h. Once grown as a monolayer, cells were incubated with increasing concentrations of C6O4 ranging from 10 ng/ml to 50 μg/ml, for 3 h. After the incubation period, the medium was removed and replaced by complete medium (K-SFM for HK-2 cells and EMEM with 10% FBS for HEK-293 cells), in the presence of 10 μg of 5-bromo-2′-deoxyuridine (BrdU). DNA synthesis was detected by incorporation of BrdU into cellular DNA, as indicated by the manufacturer's protocol (Roche Applied Science), 24 h after C6O4 removal. Cells were then fixed with 0.5 M ethanol/HCl and incubated with nuclease to digest the DNA. BrdU incorporated into the DNA was detected using an anti-BrdU peroxidase-conjugated antibody and visualized with a soluble chromogenic substrate (Roche Applied Science, Mannheim, Germany). Optical density was measured with an ELISA reader at 405 nm. In each experiment, control cultures consisted of cells maintained in complete medium without C6O4 (KSF-M for HK-2 cells and EMEM+10%FCS for HEK293 cells) and cells maintained in low-glucose DMEM without serum and without C6O4.

2.4. Uptake of E3S by human tubular cells

OAT4 has been reported to facilitate the reabsorption of conjugated steroids such as E3S with a high affinity (Cha et al., 2000; Nakagawa et al., 2009). Therefore, E3S uptake was measured in parallel in HK2 and HEK293 cells as a control, to evaluate if the increased expression of OAT4 and URAT1 influenced the absorption rate of this substrate.

HEK293 or HK-2 cells, transiently transfected with control or OAT4 or URAT1 recombinant plasmids, were seeded in 24-well plates at a concentration of 75000 cells/well in the presence of gelatin. The cells were washed twice with physiological saline to remove the serum. The medium containing 0.5 μg/ml of E3S was pre-warmed at 37 °C and added to the culture (1 ml/well). After 5 and 10 min of incubation, the medium was recovered, centrifuged at 3000 g for 5 min to remove cells and debris, filtered (0.45 μm), and the concentration of E3S was evaluated by ELISA (Invitrogen), following the manufacturer's protocol.

2.5. Uptake of C6O4 and PFC6 by human tubular cells

The uptake of C6O4 and PFC6 was evaluated with human tubular cells HEK293 and HK2 transfected with control plasmid and with plasmids allowing over-expression of OAT4 or URAT1 genes. The cellular uptake was assessed indirectly by measuring the substance concentration (C6O4 or PFC6) in the cell culture medium following incubation for 5 and 10 min.

Preliminary experiments were performed to select the best conditions to obtain transient expression of OAT4 and URAT1 in the two renal tubular cell lines. To this purpose, a control plasmid containing the GFP gene was used, and GFP expression was measured by cytofluorimetric analyses at different time points after the transfection procedure. Different commercially available transfection kits were tested. The best result (about 60% of transfected cells expressing GFP, 5 days after the transfection procedure) was obtained using Lipofectamine LTX (Life Technology), and in particular 2.5 μg of DNA and 12 μl of lipofectamine in a 6-well plate.

HEK293 and HK-2 cells, transiently transfected with control, OAT4, or URAT1 recombinant plasmids, were seeded in 24-well plates at a concentration of 75,000 cells/well. To maintain cell adhesion for all the experimental procedures, the cells were seeded in the presence of gelatin (Sigma-Aldrich, Merck Life Science S.r.l.). When cells reached the confluence, the uptake experiments were performed as reported in Suppl Table 2.

The cells were washed twice with physiological solution to remove the serum that could interfere with measurement. The medium containing 3 μg/ml of PFC6 or C6O4 was pre-warmed at 37 °C and added to

the culture (1 ml/well). After 5 and 10 min of incubation the medium was recovered.

The concentration of C6O4 for the cell uptake assay was chosen in the linear range of the analytical method calibration curve (see Section 2.6). Preliminary experiments demonstrated that this relatively high concentration did not interfere with cell proliferation in both cell types. To explore the potential interaction with cultured cells, and in the absence of available data on human exposure to C6O4, the test concentration of C6O4 and PFC6 for the in vitro experiments was selected much higher than blood or serum levels reported in the literature for various legacy PFAS monitored in the human population, including PFC6 (Poothong et al., 2017).

2.6. Chemical quantification in cell culture supernatants

Sample preparation. The medium recovered from cells incubated under different conditions (see Section 2.4 for more details) was centrifuged at 3000 g for 5 min and filtered over 0.45 μm nylon syringe filter, transferred into the suitable vials and analyzed by UPLC-MS/MS without any other pretreatment. To confirm that nylon filters (0.45 μm) did not retain the perfluorinated compounds, standard samples dissolved in cell culture medium were analyzed, before and after filtration. No filter retention was observed.

Standard preparation. C6O4 and PFC6 standard solutions were prepared diluting suitable standards in cell culture medium in order to take into account the matrix effect.

UPLC-MS/MS analysis. Quantification of analytes was carried out in MRM modality on UPLC-TQD system (Waters Acquity-TQD) using Adamas C18-X-Bond column (2.1 ×50, 1.8 μm) maintained at 40 °C. Elution was performed in gradient mode using aqueous solution of $AcONH_4$ 10 mM (A) and acetonitrile (B), at 0.200 ml/min, according to the following gradient profile: (time, B%) 0.0, 5; 6.2, 40; 7.0, 50; 8, 80; 11, 80. Mass spectrometer parameters were set as follows: Ionization ESI- (Capillary 3.90 V, Cone 16.0 V, Source temperature 120 °C, Desolvation temperature 220 °C, Desolvation gas flow 500 L/hr, Cone gas flow 50 L/hr, collision gas flow 20 ml/min.

C6O4 was quantified using the transition 338.90->179.00 (m/z) with collision energy of 5 eV; the transition 338.90-> 85.00 (m/z), with collision energy of 22 eV, was used to check the identity of the compound.

PFC6 was quantified using the transition 313.00-> 269.00 (m/z) with collision energy of 9 eV; the transition 313.00-> 119.00 (m/z), collision energy of 21 eV, was used to check the identity of the compound.

Calibration curves (Fig. S1 for C6O4 and S2 for PFC6), obtained using standard solutions, show good linearity in all operative ranges (0.20 – 16.6 μg/ml) for both compounds ($r^2 = 0.9974$ for C6O4, $r^2 = 0.9996$ for PFC6). Examples of chromatograms obtained by UPLC MS/MS analysis of supernatant samples coming from cells treated with C6O4 or PFC6 are reported in Fig. S3 and S4, respectively. The limit of quantification (LOQ) coincides with the lowest standard concentration (0.20 μg/ml) for both compounds. The limit of detection (LOD) was determined in 0.03 μg/ml for C6O4 and PFC6. The margin of error (95% confidence level) was determined as 0.20 ± 0.03 μg/ml, 2.48 ± 0.23 μg/ml for PFC6 and 0.20 ± 0.01 μg/ml, 3.10 ± 0.14 μg/ml for C6O4 over 10 injections for each point.

2.7. In silico analyses

Protein modeling. Given the absence of experimental structures for both URAT1 and OAT4, homology models were generated or retrieved, for the outward and inward form of the transporters. In particular, for URAT1, an outward model was retrieved from literature (Zhao et al., 2020), and inward conformations were obtained for URAT1 and OAT4 from AlphaFold (Jumper et al., 2021). Moreover, starting from the OAT4 primary sequence, a series of additional homology models were

generated with the I-TASSER web server (Roy et al., 2010), to obtain an OAT4 outward model.

The geometrical reliability of the identified and predicted models was evaluated through the corresponding Ramachandran plots, obtained using MolProbity webserver (Figs. S5-8) (Williams et al., 2018). Then, for the four models, the potential pockets were determined using the FLAPsite algorithm (Baroni et al., 2007), and considering data obtained in mutagenesis experiments (Zhao et al., 2020).

Before running docking simulations, hydrogens were added and H-bonds were optimized through the protein preparation wizard tool in Maestro, fixing the residue tautomeric states (Sherman et al., 2006).

Docking simulations. Induced fit docking simulations were carried out for PFOA, PFC6, E3S, and for each of the four C6O4 stereoisomers, in the four mentioned models, with Maestro (Sherman et al., 2006). The extra-precision (XP) protocol was adopted using a 12 Å docking box, a maximum number of poses equal to five and refining residues within 8.0 Å of ligands. The docking centroids were selected according to the pocket center of mass or to residues critical for anion transport.

2.8. Statistical analysis

Results from independent experiments were expressed as mean ± SD. Statistical analyses of quantitative variables were performed using analysis of variance (ANOVA), eventually followed by Tukey's or Sidak's test for multiple comparisons between groups, using the PRISM package. A p-value of < 0.05 was considered significant.

3. Results

3.1. C6O4 did not affect proliferation of renal cells

The structure of each analyzed compound is reported in Fig. 1.

The analysis of three experiments, carried out in quadruplicates for each cell line, showed that C6O4 treatment did not modify the proliferation of human tubular cells HEK293 (Fig. 2 A) or HK2 (Fig. 2B) after 24 h, up to the highest concentration of 50 μg/ml. Indeed, no statistical difference in BrdU incorporation was found in either cell type, in the presence or absence of C6O4, compared to the complete medium used as a control.

3.2. C6O4 is not significantly uptaken into human tubular cell lines

The activity of the transporters in the transfected cells was verified and compared to control plasmid-transfected cells, using estrone-3-sulfate (E3S) as a control substrate. The concentration of E3S was found decreased in treated cell supernatants, indicating that E3S was absorbed efficiently by both HEK293 and HK2 cells expressing OAT4 (2.5-fold reduction compared to cells transfected with the control plasmid). E3S concentration was also reduced in the supernatant from HEK293 and HK2 cells expressing URAT1, although to a lesser extent (1.25-fold) (Fig. 3).

Fig. 4 shows the analysis of the fluorinated compounds in supernatants from the transfected cells, following treatment for 5 or 10 min with 3 μg/ml of PFC6 or C6O4. We observed a significant reduction of PFC6 concentration in the supernatant from both the HEK293 cells transfected with control plasmid (1.4-fold), and URAT1- or OAT4-upregulated HEK293 cells (2.2-fold for each), after 5 min of incubation (Fig. 4 A). The decrease was more marked in transfected HEK293 cells that overexpress URAT1 or OAT4, compared to the cells transfected with control plasmid (Fig. 4 A). In contrast, the concentration of C6O4 was not significantly reduced in the supernatant from HEK293 cells transfected with plasmid control, or from transfected HEK293 cells over-expressing URAT1 or OAT4, after 5 and 10 min of incubation (Fig. 4 A).

Similarly, in HK2 cells, the concentration of PFC6 was significantly reduced after 5 min of incubation in both the supernatant from cells transfected with control plasmid, or from transfected cells with up-
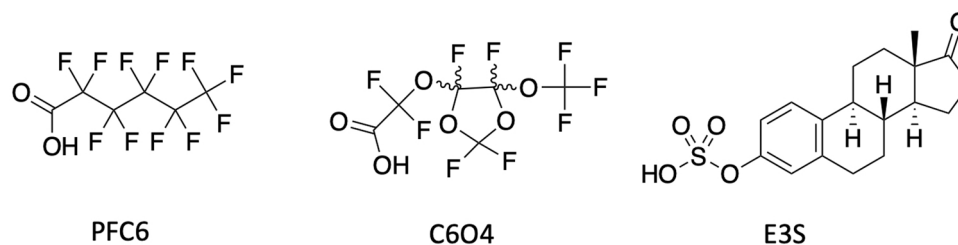
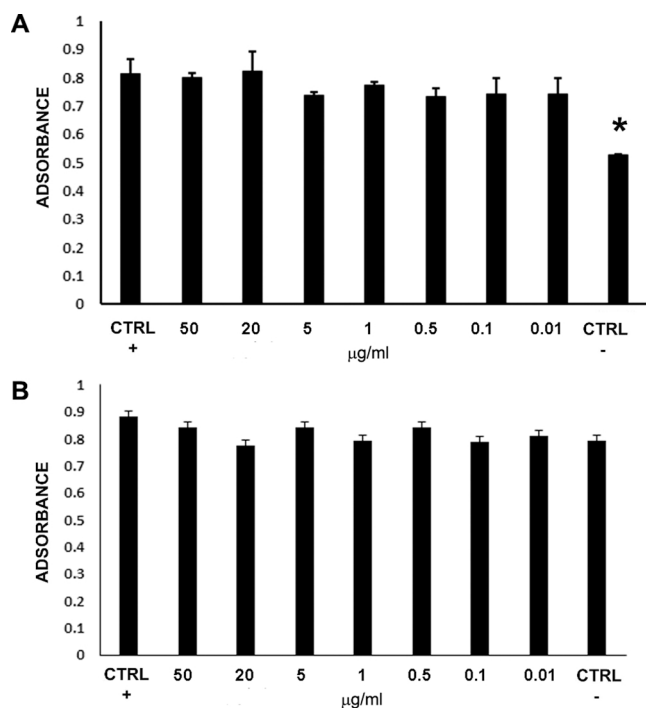**Fig. 1.** Molecular structures of the analyzed compounds.

Fig. 2. Proliferation of human tubular cells after incubation with C6O4. HEK293 (Panel A), and HEK293 (Panel B) cells have been cultured in the presence of different concentrations of C6O4 for 3 h. Cell proliferation was determined by BrdU incorporation, 24 h after C6O4 removal. Results are shown as mean $\pm$ SD of three independent experiments performed in quadruplicate. Positive control (CTRL+) was the complete medium (KSF-M for HK-2 cells and EMEM+10%FCS for HEK293 cells) and the negative control was DMEM without FCS. Statistical analysis was performed using analyses of variance (ANOVA) with Tukey's test by PRISM. *$p < 0.05$: HK2 cells maintained in DMEM without FCS compared to HK2 cells maintained in complete medium. No statistical differences were found between cells exposed to different concentrations of C6O4.

regulation of URAT1 or OAT4, (Fig. 4B). Increased expression of URAT1 or OAT4 (Fig. 4B) in transfected cells did not further enhance the PFC6 uptake. By contrast, following similar incubation periods of 5 or 10 min, the concentration of C6O4 was not significantly reduced in the supernatants from HK2 CTRL or HK2 cells with up-regulated expression of URAT1 or OAT4 (Fig. 4B).

3.3. In silico structural analyses show that URAT1 and OAT4 might poorly transport C6O4

Both URAT1 and OAT4, as other members of the OAT family, need to undergo a relevant conformational transition to transport anions from the extracellular to the intracellular medium. In particular, it has been reported (Quistgaard et al., 2016; Zhao et al., 2020) that these transporters need to switch from an inward to an occluded and outward conformation. Given the absence of experimental structures for URAT1 and OAT4, but considering the availability of homologous templates for the outward and inward conformations, the corresponding homology models were identified or generated for both transporters.

URAT1 and OAT4 models are quite similar, as well as their primary sequence (about 52% of identity). In particular, the major part of essential amino acids involved in transport are conserved (Fig. S9).

The pocket search performed with FLAPsite returned three partially connected pockets for URAT1-outward, one main pocket for URAT1-inward and OAT4-inward models. In the case of OAT4-outward, because of the identified very wide pocket, we used the midpoint between Arg473 and Arg486 as the centroid (Fig. 5). Crucial residues for ligand binding and transport are indicated in Fig. S10 (Perry et al., 2006; Zhao et al., 2020).

Induced fit docking simulations were carried out for C6O4 (4 stereoisomers), E3S, PFC6 and PFOA in all URAT1 and OAT4 models (Fig. 5). Results of PFOA docking are reported in Fig. S11 and are in agreement with those obtained for PFC6. Considering the flexible nature of the transporters and the use of homology models, we retained a certain flexibility at the binding site level (see Methods for further details). The docking scores obtained for each complex are reported in Table 1.

3.3.1. URAT1-outward model

The channel of URAT1-outward is formed by two connected pockets (1 and 3) lined by hydrophobic and positively charged residues, such as Arg477 and Arg487. The latter are located at the edges of the channel and, according to Zhao et al. (Zhao et al., 2020), are involved in guiding the anion transport through electrostatic interactions. Three different centroids were chosen for the induced fit docking simulation: the center of mass of pockets 1 (P1) and 3 (P3), and Gly361 (P2), located on a loop at the interface of P1 and P2 (Fig. 5A). This residue seems to be as well crucial for the transporter activity, because of the flexibility conferred to the loop and its low steric hindrance.

The first induced fit docking in P1 resulted in the interaction of the ligands with Arg487, through the formation of a salt bridge and H-bond interactions (Figs. S11A and S12A, B, C). C6O4 showed higher values, with a slight and a more significant increase compared to the scores obtained by PFCAs and E3S, respectively. Since the score corresponds to a free energy approximation, the lower the value, the more stable the interaction is. The docking performed in P2, close to Gly361, returned similar poses for PFCAs and E3S, but different for C6O4. PFC6 and PFOA interact with Arg477, reaching the lower P3 and forming additional hydrophobic contacts with the lining residues (Fig. 6 A, and Fig. S11B). Differently, the majority of the C6O4 poses, and E3S, are placed in the upper and more external P1, where they contact Arg487 (Fig. 6B, C). This could be due to the higher linearity and hydrophobicity of PFCAs and E3S compared to the bulkier C6O4 isomers, likely less prone to move along the transport channel. Docking scores are significantly different too, with the C6O4 values being about 3 units higher than both PFCAs and E3S scores (Table 1). Docking simulations carried out in P3 returned similar poses for all ligands but, again, more favorable scores for PFCAs and E3S, which better fit this hydrophobic cavity (Figs. S11C and S12D, E and F).
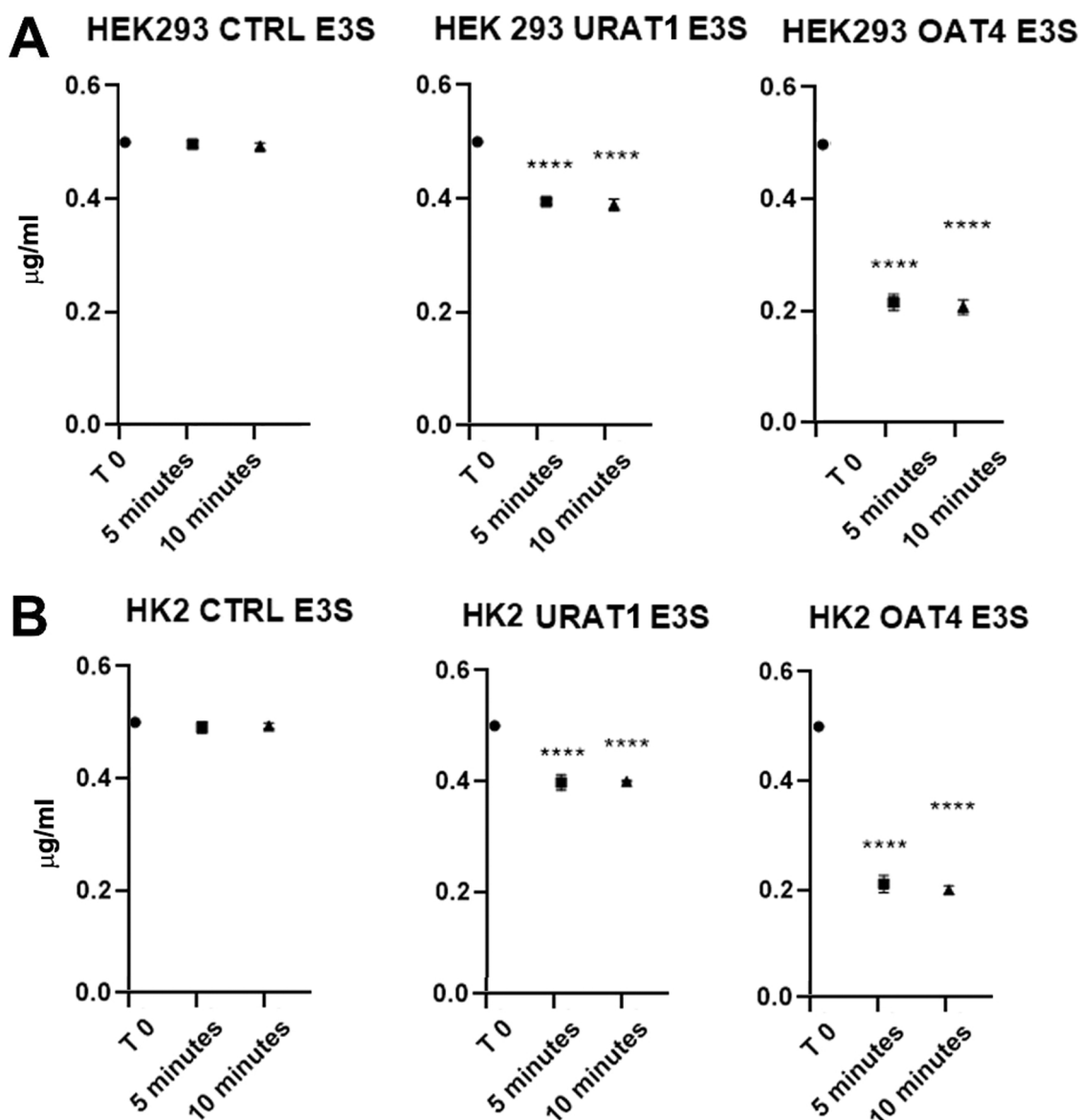
Fig. 3. Uptake of a control substrate (estrone-3-sulfate) by human tubular cells transfected with control or with URAT1 or OAT4 plasmids. Concentration of E3S (μg/ml) in the supernatants from HEK293 (panel A) and HK2 (panel B) cells transfected with control plasmid (CTRL), or with URAT1 or OAT4 plasmids, at basal level and after 5 and 10 min of incubation at 37 °C. Data are presented as mean ± SD of three independent experiments performed in duplicate (n = 6). Statistical analysis was performed using analyses of variance (ANOVA) with Tukey's test by PRISM. * ** *p < 0.0001 versus T0.

3.3.2. URAT1-inward model

The transporter presents a unique large hydrophobic cavity, again lined by some positively charged and hydrogen-bond donor residues (Arg349, Lys393, Gln473 and Arg477; Fig. 5B). In all docking simulations the ligands interact with Arg477 and Gln473 (except for PFC6, only forming a salt-bridge with Arg477, Fig. 6D). The poses are quite different between PFCAs and C6O4 isomers, indeed, while PFCAs are located in an upper and narrower cavity lined by aromatic residues (Fig. 6D and Fig. S11D), bulkier C6O4 isomers are placed below Gln473 and Arg477 (Fig. 6E). The large score difference supports a better transport of PFCAs by URAT1 compared to C6O4, as also shown by in vitro experiments. E3S maintains the same interactions with Gln473 and Arg477 and forms an additional contact with Lys393 (Fig. 6F). The docking score is similar to that of PFCAs, and much more favorable than those obtained with C6O4.

3.3.3. OAT4-outward model

The model obtained from I-TASSER is slightly different with respect

to URAT1, and presents a single large pocket facing the extracellular space (Fig. 5C). Docking simulations were performed using a centroid, the midpoint between Arg473 and Arg486, corresponding to Arg477 and Arg487 in URAT1 (see sequence alignment, Fig. S9). The docking poses are quite similar for all ligands, mostly interacting with Arg389 (Fig. 6 G, H, I, and Fig. S11E), but scores are significantly higher for C6O4 than for the PFCAs and E3S, which also form pi-pi contacts with Tyr361.

3.3.4. OAT4-inward model

This model, retrieved from AlphaFold, is almost identical to URAT1-inward (Fig. 5D). The only difference is associated with a reduced presence of hydrophobic residues in the OAT4 pocket. Residues as Trp357, Phe360 and Phe449 in URAT1 are respectively replaced by polar Asn353 and smaller Leu356 and Leu345, making the OAT4 pocket more polar and flexible. Docking simulations, performed using as centroid the center of mass of the pocket identified by FLAPsite, provided less conserved poses, with PFCAs and C6O4 interacting with an
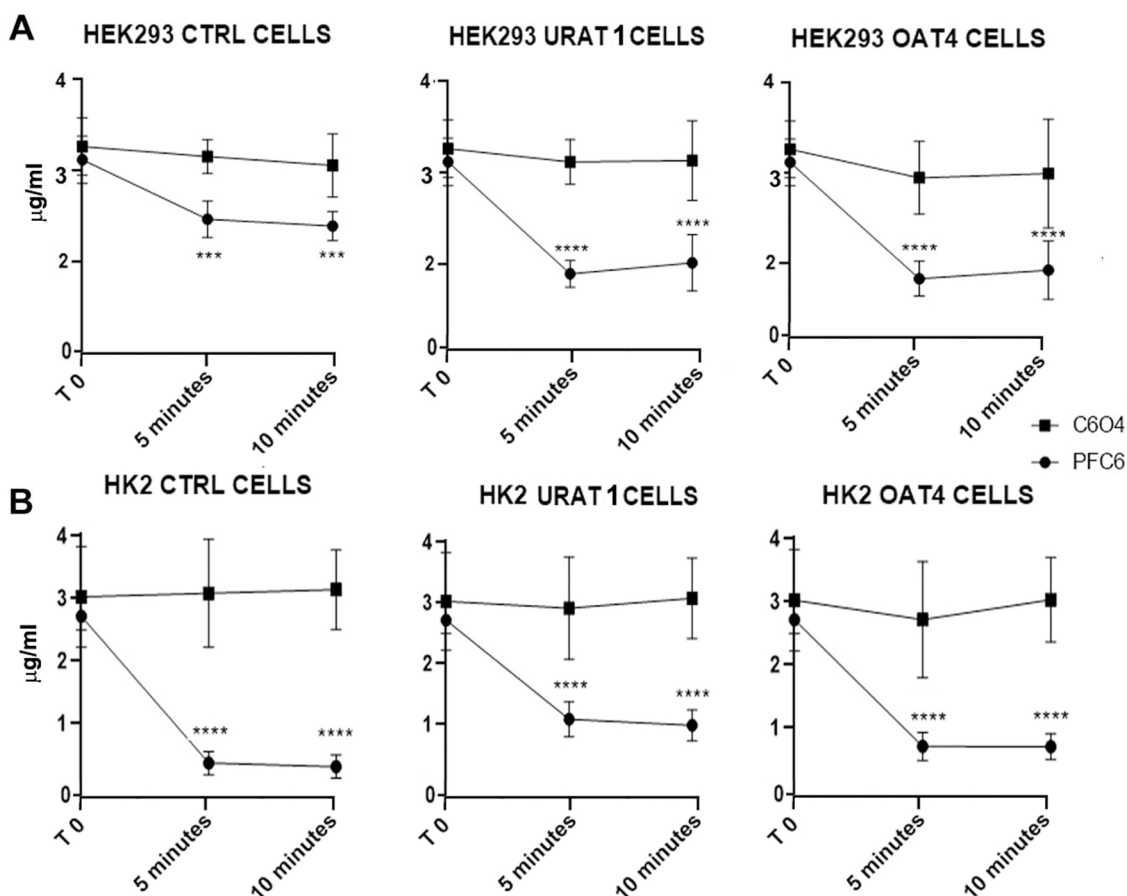
Fig. 4. Uptake of C6O4 and PFC6 by human tubular cells transfected with control or with URAT1 or OAT4 plasmids. A) Concentration of C6O4 and PFC6 (µg/ml) in the supernatants from HEK293 cells transfected with control plasmid (CTRL), or with URAT1 or OAT4 recombinant plasmids, at basal level and after 5 and 10 min of incubation at 37 °C. Data are presented as mean ± SD of three independent experiments performed in duplicate (n = 6). Statistical analysis was performed using the Two-way ANOVA with Sidak's multicomparison test by PRISM. * **p < 0.0002 concentration of PFC6 vs C6O4 for CTRL cells and * ** *p < 0.0001 concentration of PFC6 vs C6O4 for OAT4 and URAT cells. B) Concentration of C6O4 and PFC6 (µg/ml) in the supernatants from HK2 cells. transfected with control plasmid (CTRL), or with URAT1 or OAT4 plasmids, at basal level and after 5 and 10 min of incubation at 37 °C. Data are presented as mean ± SD of three independent experiments performed in duplicate (n = 6). Statistical analysis was performed using the Two-way ANOVA with Sidak's multicomparison test by PRISM. * ** *p < 0.0001 concentration of PFC6 vs C6O4 for CTRL, OAT4 and URAT HK2-cells.

ensemble of several basic and polar residues (Tyr360, Arg389, His469, Arg473, and Asn353 among others; Fig. 6 J,K and Fig. S11F). E3S interacts only with Asn353 and Lys452 respectively through an H-bond and a salt-bridge, and forms a cation-pi interaction with Arg473 (Fig. 6 L).

Docking scores of C6O4 isomers are again lower than the ones for PFCAs and E3S, but with a smaller difference compared to that obtained for URAT1-inward.

4. Discussion

In this study, in vitro exposure of two human kidney cell lines to C6O4 did not affect cell proliferation, even at relatively high exposure concentrations. This result is in agreement with those reported on cultured thyroid cells from rats and humans, in which C6O4 did not significantly alter cell proliferation parameters or cause detectable toxicity (Coperchini et al., 2021a).

Previous work by Yang et al., 2010, showed that linear PFCAs are substrates of OAT4 and URAT1, with varying levels of affinity depending on their chain length. This suggests that OAT4 and URAT1 are key transporters in the renal reabsorption of linear PFCAs, and possibly explains the long half-life of these compounds in humans (Yang et al., 2010).

The present study was set to investigate C6O4 transport potential

into renal cells. To determine whether the same apical transporters were involved, the uptake of C6O4 was measured using human renal tubular cells transfected with control plasmid, or the same cells transfected with the URAT1 or OAT4 human genes.

Experiments using E3S as a known substrate for human OAT4 provided evidence of the expected activity of OAT4 and URAT1 transporters in the transfected human renal cells HK2 and HEK293.

The uptake experiments following treatment with PFC6 or C6O4 showed marked differences. The concentration of PFC6 was found significantly decreased in the supernatant from plasmid control-transfected cells, as well as from transfected HEK293 cells and HK2 cells overexpressing URAT1 or OAT4 genes, supporting a clear uptake of PFC6 into those renal cells. This is consistent with previous findings reported for PFC6 (Yang et al., 2010) and PFOA (Nakagawa et al., 2009). In contrast, the concentration of C6O4 in the supernatants was not significantly modified in all the test conditions, even after 10 min of incubation, indicating that no substantial absorption into the renal cells had occurred.

Thus, our results show that, contrary to linear PFCAs, there does not seem to be a significant reuptake of C6O4 into human renal cells through an active transport mechanism mediated by URAT1 or OAT4, while the small decrease observed in the supernatant of control cells could be linked to some level of passive diffusion over time. This may indicate a low affinity of C6O4 for these human transporters, possibly related to
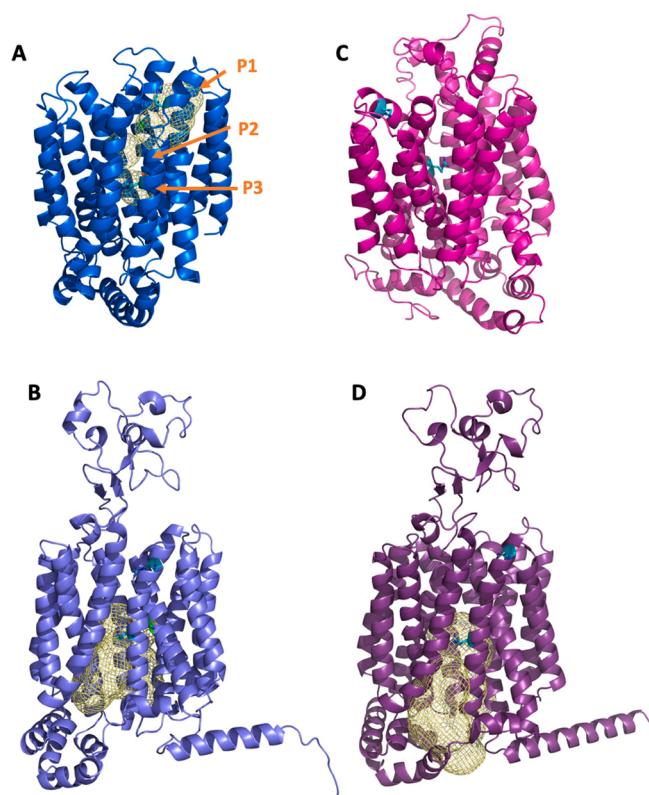
Fig. 5. Homology models and pockets used for docking simulation. Residues crucial for binding and transport are shown in capped sticks. The protein is displayed as a cartoon, the pocket as a dashed surface. A) URAT1-outward. B) URAT1-inward (docking has been performed using the midpoint between Arg473 and Arg486 as centroid. C) OAT4-outward; D) OAT4-inward.

Table 1
Glide docking scores for C6O4, E3S, PFC6 and PFOA in the four models.

Model	Pocket	Induced fit Glide score			
		PFC6	PFOA	C6O4[a]	E3S
URAT1-outward	1	-6,0	-6,1	-5,6 ± 0,9	-10,4
	2	-8,5	-9,6	-6,1 ± 1,0	-9.0
	3	-10,0	-10,2	-8,3 ± 0,6	-10,0
URAT1-inward		-8,9	-10,0	-6,2 ± 0,9	-9,2
OAT4-outward		-7,9	-8,4	-6,6 ± 0,8	-8,8
OAT4-inward		-8,6	-9,5	-8,2 ± 0,6	-8,7

[a] The score corresponds to the average ± SD value for the four stereoisomers.

the cyclic structure and lower lipophilicity of the molecule, with respect to linear PFCAs. Considering the obtained results, it can be inferred that these two apical transporters do not participate in the active reabsorption of C6O4 from the proximal tubule. This contrasts with what is reported for linear PFCAs in human and rat cells (Nakagawa et al., 2009; Weaver et al., 2010; Yang et al., 2010).

In silico modeling demonstrated that the in vitro activity correlated well with the predicted interaction of the two transporters with PFCAs, C6O4 and E3S. In particular, the differential binding of PFOA and PFC6 to OAT4, with a lower binding predicted for PFC6 compared to PFOA (Table 1), is consistent with recent molecular docking evaluations supporting that the binding affinities of PFCAs for OAT4 were dependent on their chain length (Lu et al., 2021; Ma et al., 2021). However, this is the first study assessing the interaction of C6O4 with OAT4 and URAT1 transporters. A low affinity of C6O4 for these proteins would mean a lower concern for active potential uptake into tissues and cell types expressing these transporters. The simulations also allowed the identification of a possible molecular path along these two transporters for the

reuptake of anions. Indeed, the transporter internal channel is lined by numerous hydrophobic residues, and a few positively charged residues that might provide the electrostatic driving force for the transport. Additional *in silico* simulations using molecular dynamics and free energy calculations could be performed to further explore the flexibility, the transport mechanism and the interaction energy of PFCAs and C6O4 towards URAT1 and OAT4.

As the present study suggests no active uptake of C6O4 from urine would delay its renal elimination, alternative pathways remain to be explored. Other mechanisms, such as serum protein binding and nuclear receptor activation, are known to influence the systemic distribution, toxicokinetics and renal elimination of PFCAs, and might be relevant to C6O4 (Houck et al., 2021; Ng and Hungerbühler, 2013). Serum albumin is the most abundant hormone carrier protein in vertebrates and can contribute to systemic distribution of particular PFCAs according to their binding affinities (Chen and Guo, 2009; Forsthuber et al., 2020; MacManus-Spencer et al., 2010). In humans, some PFCAs have also been shown to bind the other less abundant carrier proteins such as Transthyretin (TTR) and Thyroxine-binding protein (TBG), but with a high affinity which can lead to displacement of endogenous hormones, especially thyroxine (T4) (Coperchini et al., 2021a, 2021b; Ren et al., 2016; Weiss et al., 2009). Binding has been also suggested towards the ligand binding domain of the peroxisome proliferator-activated receptor gamma (Li et al., 2019; Zhang et al., 2014), for some cytochrome P450 isoforms (Amstutz et al., 2022; Narimatsu et al., 2011) and for liver fatty acid binding proteins (Zhang et al., 2013). Different computational approaches have already proven to be effective for the affinity prediction of new PFCA targets (Cheng and Ng, 2018; Ng and Hungerbühler, 2013), thus, the development of computational modeling could provide a useful tool to predict potential interactions of C6O4 with these proteins.

Interestingly, fluorinated compounds affect differentially cultured thyroid cells, according to their structure (Croce et al., 2019). Similarly, Coperchini et al. (2021a, 2021b) investigated cytotoxicity, necrosis/apoptosis, the formation of reactive oxygen species and observed no particular adverse effects of C6O4 on cultured rat thyroid cells FRTL-5 and primary cultures of normal human thyroid cells, contrary to the long-chain PFOA and PFOS. Further investigations are necessary to understand the behavior of this unique structure.

5. Conclusions

Altogether, the low interaction potential with the human transporters OAT4 and URAT1 combined with the lack of uptake by renal cells expressing these transporters suggest renal proximal tubule reabsorption is not likely to play a role in the renal elimination of C6O4.

CRediT authorship contribution statement

Stefania Bruno: Funding acquisition, Conceptualization, Investigation, Writing – original draft, **Matteo Bersani:** Investigation, Writing – original draft, Validation, **Serena Astore:** Methodology, Investigation, **Giulia Chiabotto:** Methodology, Investigation, Validation. **Alessandro Barge:** Investigation, Conceptualization, Writing original draft, **Arianna Binello:** Investigation, Methodology, **Francesca Spyrakis:** Conceptualization, Formal analysis, Funding acquisition, Writing – review & editing.
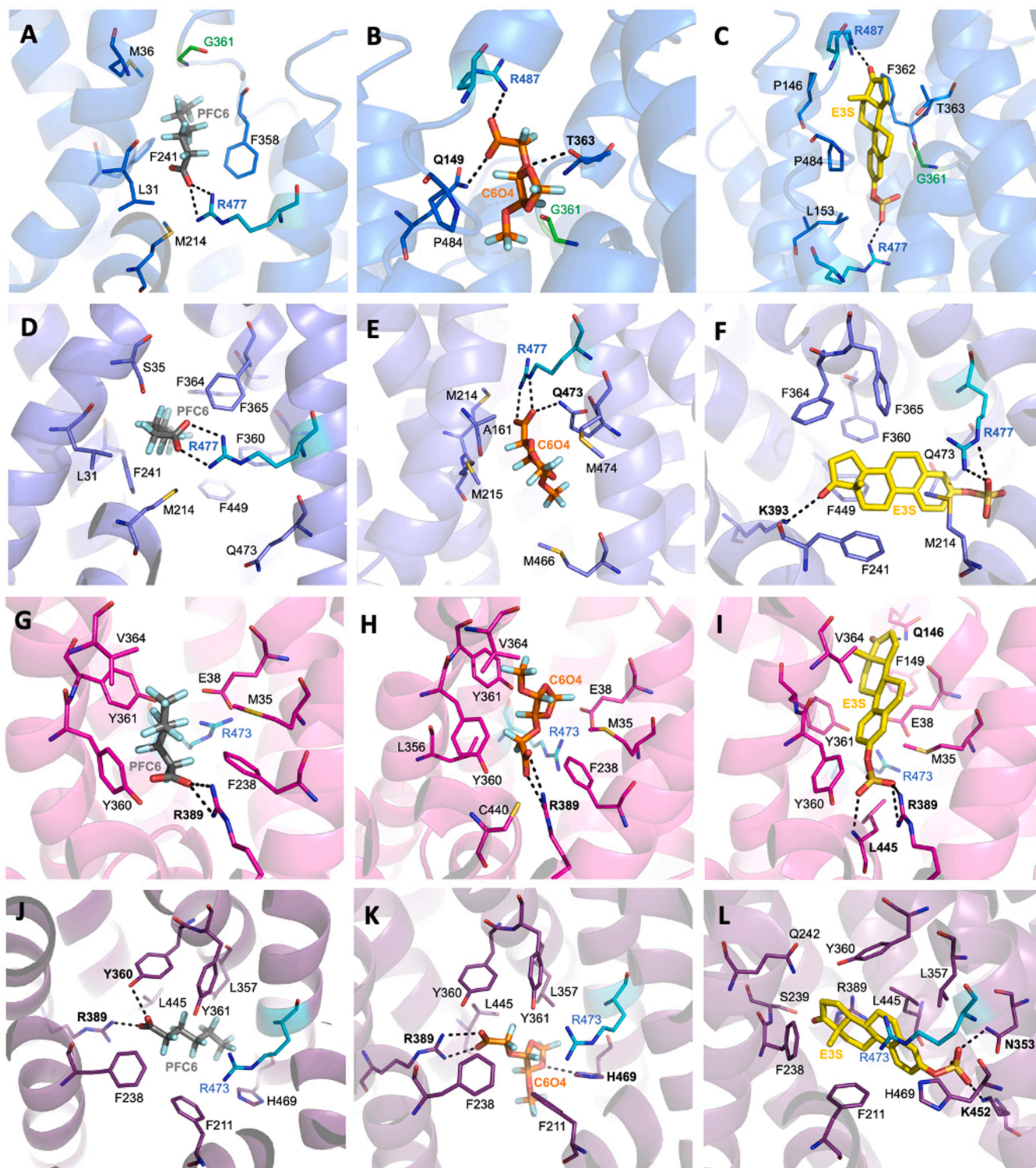
Fig. 6. Docking poses of the analyzed compounds. Docking poses of PFC6 (grey color-code sticks), C6O4 (orange color-code sticks) and E3S (yellow color-code sticks) in URAT1-outward pocket 2 (blue cartoons), URAT1-inward (violet cartoons), OAT4-outward (magenta cartoons) and OAT4-inward (purple cartoons). Specifically: A. PFC6 in URAT1-outward P2. B. C6O4 in URAT1-outward P2. C. E3S in URAT1-outward P2. D. PFC6 in URAT1-inward. E. C6O4 in URAT1-inward. F. E3S in URAT1-inward. G. PFC6 in OAT4-outward. H. C6O4 in OAT4-outward. I. E3S in OAT4-outward. J. PFC6 in OAT4-inward. K. C6O4 in OAT4-inward. L. E3S in OAT4-inward. Residues lining the pocket are shown in sticks, interacting residues are bold labeled, and key residues for transport are colored differently with respect to the corresponding transporter. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Declaration of Competing Interest

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2022.153257.

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