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Short Communication

A Novel Human Organic Anion Transporter NPT4 Mediates the Transport of Ochratoxin A

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Abstract. In the present study, we investigated the transport of nephrotoxic mycotoxin ochratoxin A (OTxA) by a novel human organic anion transporter hNPT4 using the *Xenopus* oocyte expression system. hNPT4 mediated time- and concentration-dependent uptake of OTxA (K_m: 802.8 μM) in a pH- and voltage-sensitive manner. *Cis*-inhibition experiments suggest that the substrate selectivity of hNPT4 is similar to that of hOAT4. The fact that the K_m of OTxA for the efflux transporter hNPT4 was much higher than those for the uptake transporters hOAT1 and hOAT3 may favor the accumulation of OTxA in the tubular cell and lead to nephrotoxicity.

Keywords: ochratoxin A, organic anion transporter, nephrotoxicity

Ochratoxin A (OTxA), a naturally occurring mycotoxin produced as a secondary metabolite in different species of *Aspergillus* and *Penicillum* (1), has been receiving considerable attention because of its deleterious effects on human and animal health (2). OTxA induces oxidative DNA damage and causes nephrotoxicity in experimental models and is suspected to be one of the causal toxins for Balkan endemic nephropathy, a chronic tubulointerstitial disease prevalent in Balkan countries (3, 4). Within the body, OTxA accumulates in several tissues, especially in the kidney and liver, and is excreted mainly from the kidney (5). An in vivo study revealed that probenecid (a typical inhibitor of the renal organic anion transporters) decreased the renal clearance of OTxA (6). Studies using isolated renal tubules showed that the organic anion transport system mediates the uptake of OTxA and may play a role in OTxA toxicity (6). Thus, the excretion of OTxA into urine is likely to be mainly by tubular secretion, presumably via the organic anion transport system (7).

The excretion of numerous organic anions, including endogenous metabolites and drugs as well as toxic xenobiotics, is an important physiological function of renal proximal tubules (6, 8, 9). The process of excreting organic anions through proximal tubular cells is achieved via unidirectional transcellular transport, involving the uptake of organic anions into cells from the blood across the basolateral membrane, followed by extrusion across the apical membrane into the tubular lumen. To date, four isoforms of human organic anion transporters (hOATs: hOAT1 to 4) have been identified in the kidneys (6). Since OTxA has anionic moieties (1), it is classified as an organic anion and requires transporters for membrane permeation. Our group has already elucidated that among the renal hOATs, hOAT1 and hOAT3 play major roles in the basolateral uptake of OTxA in proximal tubules and hOAT4 contributes to OTxA reabsorption at the apical side of proximal tubules using the outward directed dicarboxylate gradient in the proximal tubules (10 – 12). However, how OTxA is secreted into the proximal tubular lumen remains unknown (7).

Just recently, we identified an orphan transporter, NPT4 (*SLC17A3*), classified as a type I sodium-phosphate cotransporter, that serves as a voltage-driven organic anion efflux transporter at the apical side of renal proximal tubules (13). NPT4 mediates the efflux of drugs such as *para*-aminohippurate (PAH) and diuretics and that of endogenous substrates such as urate in a voltage-sensitive manner. Since the substrate specificity of NPT4 resembles those of OAT1 and OAT3, we examined whether OTxA is a transport substrate of NPT4, a candidate transporter for the exit path of OTxA at the apical membrane.

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Human NPT4 (hNPT4) cDNA was obtained as previously reported (13). cRNA synthesis and uptake measurements were performed as previously described (14). Briefly, the cDNA was linearized with *Xba* I, and the cDNA insert transcribed in vitro with the T7 mMESSAGE mMACHINE cRNA synthesis kit (Ambion, Austin, TX, USA). Defolliculated *Xenopus* oocytes (stage IV and V) were injected with 20 ng of capped hNPT4 cRNA and incubated at 18°C in a modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 10 mM HEPES) containing gentamicin (50 μg/ml). After incubation for 2 – 3 days, uptake experiments were performed at room temperature in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) or other uptake solution as described in the figure legend. The transport experiment was initiated by replacing the uptake solution with that containing radiolabeled [^{3}H]OTxA (American Radiolabeled Chemicals, St. Louis, MO, USA) and was terminated by adding ice-cold

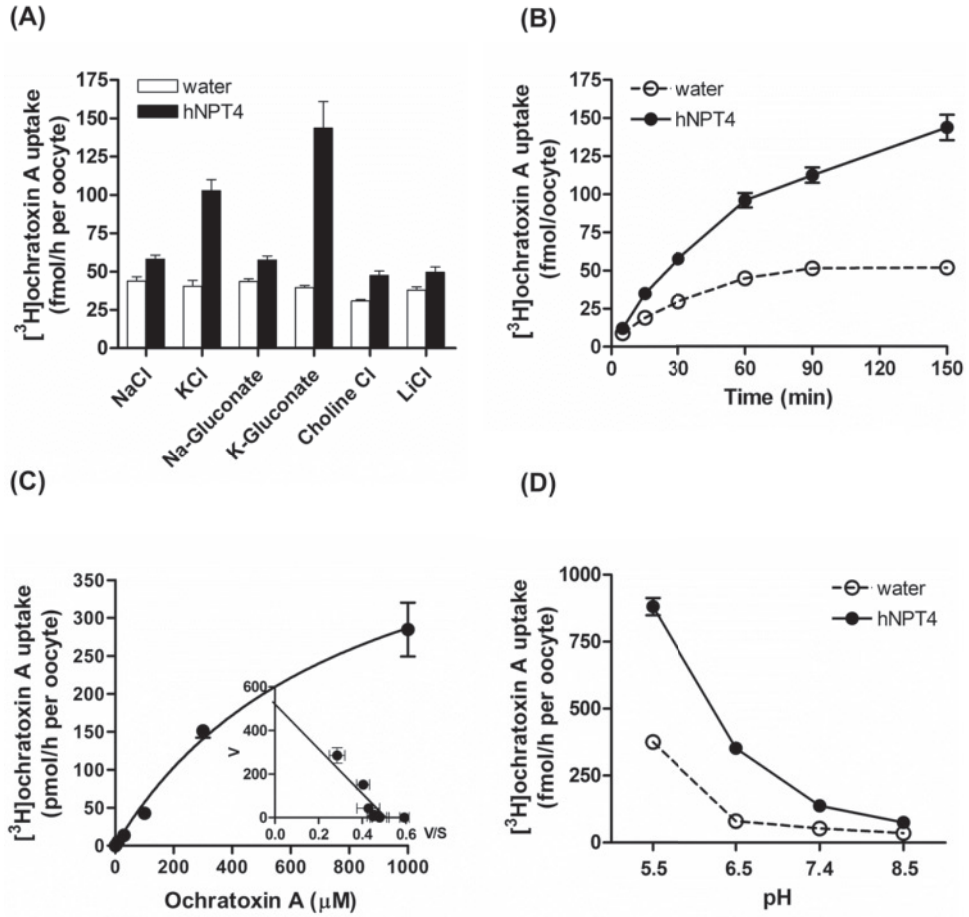


Fig. 1. Functional analysis of hNPT4-mediated [^{3}H]ochratoxin A (OTxA) uptake in the *Xenopus* oocyte expression system. A) The uptake of 0.1 μM [^{3}H]OTxA in bath solution with various ion compositions for 1.0 h in water (open bars)- and hNPT4 (filled bars)-injected oocytes. The NaCl in basal uptake solution was replaced by KCl, Na-gluconate, K-gluconate, choline Cl, and LiCl. In the case of using Na-gluconate or K-gluconate, the chloride-free condition was prepared by substituting gluconate for chloride. B) Time course of OTxA uptake by hNPT4 in high-potassium uptake solution. The uptake of 0.1 μM [^{3}H]OTxA in water-injected (control) oocytes (open circles) and hNPT4-expressing oocytes (closed circles) was measured during a 150-min incubation. C) Transport kinetics of hNPT4-mediated OTxA uptake in high-potassium uptake solution. [^{3}H]OTxA transport was measured in control oocytes and hNPT4-expressing oocytes over an OTxA concentration range of 0.3 – 1,000 μM. The hNPT4-specific transport activity in hNPT4-expressing oocytes (closed circles) was calculated by subtracting the transport activity in control oocytes. These hNPT4-specific transport activities were used in kinetic analysis. Inset, Eadie–Hofstee plot. D) The pH dependence of hNPT4-mediated OTxA transport. The [^{3}H]OTxA (0.1 μM) transport rate was measured in control oocytes (open circles) and hNPT4-expressing oocytes (closed circles) in high-potassium uptake solution. To prepare uptake solutions with different pH levels, MES-Tris (5.5), HEPES-Tris (6.5 and 7.4), and Tris-HCl (8.5) were used as the buffer systems. At acidic extracellular pH values in high potassium uptake solution, the net OTxA uptake rate was increased. The data are reported as the mean ± S.E.M., n = 8 – 10.

ND96 solution after 60 min of incubation. Oocytes were washed five times with ice-cold ND96 solution, solubilized with 5% SDS, and the radioactivity content was determined. For the inhibition study, the uptake rates of 0.1 μM [^{3}H]OTxA by oocytes injected with water or NPT4 cRNA were measured for 60 min in the absence or presence of test compounds in high potassium uptake solution.

The experiments were performed using three different batches of oocytes, and the results from the experiments were each expressed as the mean ± S.E.M. Statistical significance was judged from Student's *t*-tests. Differences were considered significant at a level of $P < 0.05$.

First, we examined the transport of OTxA in various ionic conditions to clarify its driving force. As shown in Fig. 1A, elimination of extracellular Na^+ did not affect OTxA transport by NPT4, indicating no sodium-coupled transport property. NPT4-mediated OTxA transport was sensitive to membrane potential because the elevation of external K^+ (which depolarizes the plasma membrane of a *Xenopus* oocyte) facilitated OTxA transport similar to the cases of PAH, bumetanide, and urate as previously reported (13). This voltage sensitivity should favor OTxA exit from the tubular cells because of a net negative charge within the cell, although we could not demonstrate the efflux of radiolabelled OTxA injected directly into the oocytes due to its high degree of hydrophobicity (data not shown). We then measured the effect of an outward

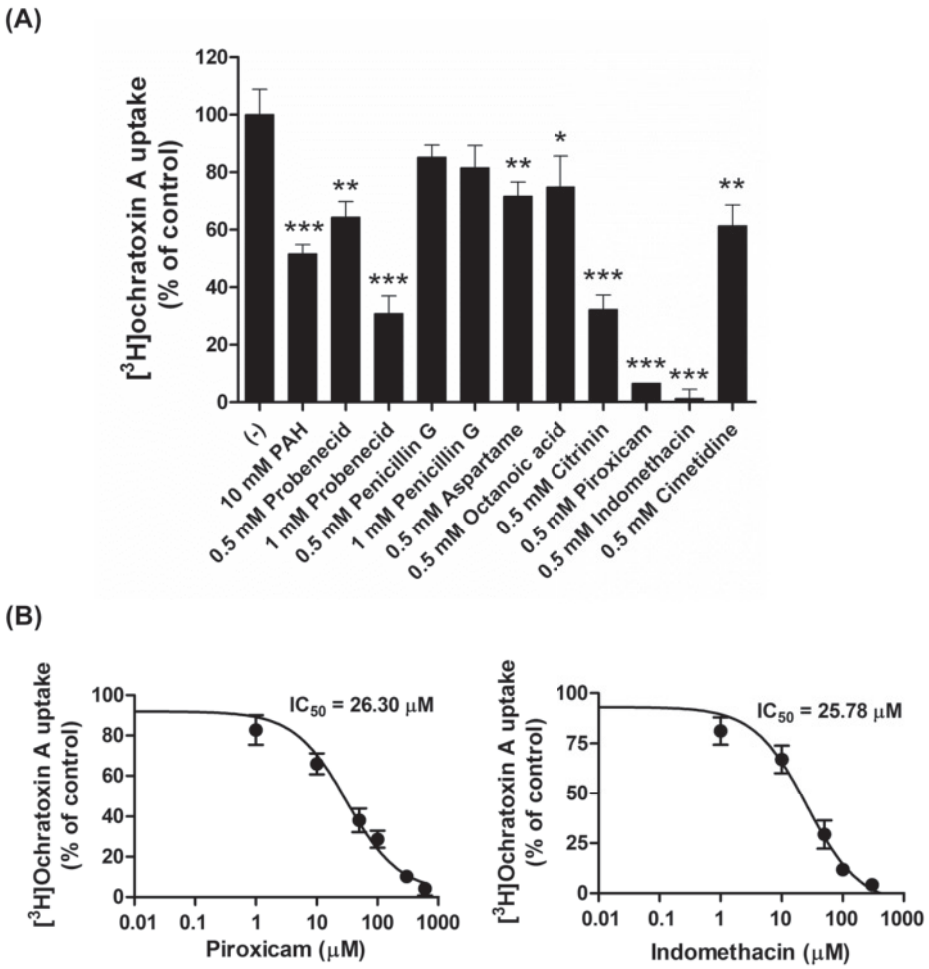


Fig. 2. Inhibition study of hNPT4-mediated [^{3}H]ochratoxin A (OTxA) uptake. Oocytes were injected with 20 ng of hNPT4 cRNA and maintained for 3 days at 18°C. A) Inhibition by various compounds (0.5, 1, or 10 mM) against 0.1 μM [^{3}H]OTxA uptake for 1 h at room temperature. The results show the values after subtraction of the uptake in noninjected oocytes from that in hNPT4-injected ones. Values represent means ± S.E.M. of 7 to 10 determinations. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. control. B) The IC_{50} test of piroxicam (left) and indomethacin (right) on hNPT4-mediated [^{3}H]OTxA uptake. Control oocytes and oocytes expressing hNPT4 were incubated with 0.1 μM [^{3}H]OTxA in the presence of 0, 1, 10, 50, 100, 300, and 600 μM piroxicam and 0, 1, 10, 50, 100, and 300 μM indomethacin for 1 h at room temperature. The data are the percentage of subtracted values of [^{3}H]OTxA uptake in noninjected ooyctes from that in hNPT4-injected ones in which the uptake in the absence of piroxicam or indomethacin equals to 100%. Values represent means ± S.E.M. of 6 determinations.

Cl^- gradient (intracellular to extracellular) on OTxA transport. Imposing a Cl^- gradient by complete removal of external Cl^- did not accelerate OTxA uptake via NPT4, indicating that NPT4 does not have the exchange mechanism for inorganic Cl^- similar to the cases of PAH, bumetanide, and urate (13).

Then, we examined the OTxA transport properties of NPT4 in the high-K^+ (depolarized) condition. *Xenopus* oocytes expressing NPT4 exhibited time- and concentration-dependent transport of OTxA (Fig. 1: B and C). The Eadie-Hofstee plot yielded a K_m of 802.8 ± 137.3 μM and a V_{max} of 518.7 ± 76.4 fmol/h per oocyte (mean ± S.E.M. of three separate experiments, respectively) for OTxA, indicating that NPT4 mediates low affinity transport for OTxA (Fig. 1C). It is notable that this K_m for NPT4 is much higher than those for other OTxA uptake transporters hOAT1 (0.42 μM), hOAT3 (0.75 μM), and hOAT4 (22.9 μM) (10, 11).

OTxA transport by NPT4 was stimulated by decreasing the extracellular pH from 8.5 to 5.5 (Fig. 1D). The rate of OTxA transport in NPT4 cRNA–injected oocytes increased by more than 8-fold when the extracellular pH was lowered from 8.5 to 5.5, although there was also an increase in the rate of OTxA transport in water-injected oocytes. These data indicate a marked pH dependence of NPT4-mediated OTxA transport. This pH-driven property of hNPT4 suggests that the majority of OTxA efflux via hNPT4 occurs towards the end of the proximal tubule, as micropuncture experiments showed the luminal pH is 7.4 in the early proximal tubule, while it is lower in the late portion (15).

The pharmacological characteristics of NPT4-mediated OTxA transport were further clarified by using various drugs, which are considered as a substrate for OATs and some of which were shown to prevent the nephrotoxicity of OTxA or inhibit the transport of OTxA (6, 10, 11). As shown in Fig. 2A, compounds (0.5 to 10 mM) tested here inhibited the [^{3}H]OTxA (0.1 μM) uptake at different ratios: piroxicam and indomethacin inhibited strongly; probenecid (1 mM) and citrinin, moderately; and aspartame, octanoic acid, and cimetidine, weakly. This inhibitory profile of hNPT4 is more similar to that of hOAT4 (except citrinin) (11) than those of hOAT1 and hOAT3 (10), suggesting that the substrate selectivity of hNPT4 is similar to that of hOAT4. Among these drugs, piroxicam and indomethacin inhibited strongly OTxA transport and hNPT4 does not seem to be the potential target of NSAIDs for the nephrotoxicity because of its low affinities for these drugs (IC_{50} values: 26.3 μM for piroxicam and 25.8 μM for indomethacin) (Fig. 2B).

To date, it was reported that the multidrug resistance protein MRP2 (*ABCC2*), localized to the apical membrane of proximal tubules in human and rat kidney, mediated the transport of OTxA in in vitro (6). They reported that OTxA inhibited MRP2-mediated PAH transport with an IC_{50} value of 58 μM. This value is also much higher than OTxA's K_m for hOAT1 and hOAT3 (10) as we mentioned above. Thus, OTxA seems to be a good transport substrate for OATs at the basolateral side of renal proximal tubules, but not efficiently secreted into the tubular lumen at the apical side because OTxA is a poor substrate for apical OATs such as NPT4 as well as MRP2. These differences between apical and basolateral OATs will lead to the accumulation of OTxA in the proximal tubular cells and OTxA may reach toxic concentrations (Fig. 3).

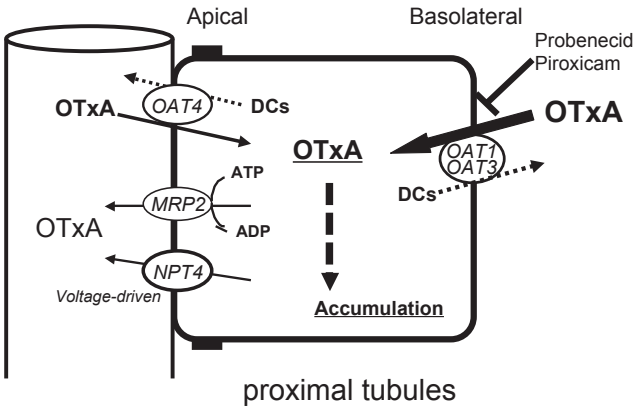


Fig. 3. Proposed model of ochratoxin A (OTxA) uptake by renal proximal tubular cells. OTxA is a good substrate for the basolateral uptake transporters hOAT1 and hOAT3 as well as apical uptake transporter hOAT4 and a poor substrate for the apical secretory transporter hNPT4 and MRP2, leading to the high intracellular concentrations and nephrotoxicity. The thickness of the arrows correlates with the extent and efficiency of transport. DCs, dicarboxylates.

In conclusion, our data show that a nephrotoxic mycotoxin OTxA transport is mediated by the novel human organic anion transporter hNPT4 in in vitro. Its low affinity to possible efflux transporters (i.e., hNPT4 and/or MRP2) may prevent efficient removal of OTxA from the proximal tubular cell, explaining in part why OTxA is nephrotoxic.

Acknowledgments

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