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Response to "Tenofovir Disoproxil Fumarate Is Not an Inhibitor of Human Organic Cation Transporter 1"

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On the basis of experiments reported in their letter, Shen and coworkers concluded that tenofovir disoproxil fumarate (TDF) is not an inhibitor of human organic cation transporter 1 (OCT1). They expressed their concern that high-affinity inhibition of the uptake of *N*-methyl-4-phenylpyridinium (MPP^+) in OCT1-expressing Chinese hamster ovary (CHO) cells observed earlier (Minuesa et al., 2009) was the result of an experimental artifact. In response, we would like to emphasize that the measurements reported by Shen and coworkers do not justify such a general conclusion, because they were performed under experimental conditions that did not necessarily allow detection of the inhibition of OCT1 by TDF. In drawing their conclusion, Shen and coworkers did not consider data concerning the complex functional mechanism of polyspecific organic cation transporters and that OCT1 contains high-affinity cation binding sites that are supposed to modulate transport and affinities of inhibitors (Gorbunov et al., 2008; Koepsell, 2011, 2015; Nies et al., 2011). Shen and coworkers measured OCT1-mediated uptake under different experimental conditions and investigated effects of TDF and MPP^+ uptake by using a 80-fold higher concentration of MPP^+ for the uptake measurements. Whereas Shen and coworkers measured uptake of 1 μM MPP^+ in confluent cell layers after incubation for 1 minute or longer, we measured uptake of 0.0125 μM MPP^+ in dissociated cells using an incubation time of 1 second.

We would first like to respond to the concerns raised by Shen and coworkers about whether OCT1-mediated MPP^+ uptake expressed in CHO cells or nonspecific uptake of MPP^+ had been determined in our 1-second uptake measurements. In CHO cells stably transfected with human OCT1 the uptake of 1 μM [^{3}H]MPP^+ was 10-times higher compared with CHO cells that were stably transfected with an empty vector (Fig. 1). Whereas the uptake of [^{3}H]MPP^+ in OCT1-expressing CHO cells was 95% inhibited when 5 mM nonradioactive MPP^+ was present during the incubation, [^{3}H]MPP^+ uptake in mock-transfected CHO cells was not significantly decreased by 5 mM nonradioactive MPP^+. The data confirm that high-affinity inhibition of TDF and the other compounds, as described previously, are attributable to inhibition of OCT1-mediated transport.

The different assay conditions employed by Shen and coworkers may have contributed to the failure to observe inhibition of OCT1-mediated uptake by TDF, because in confluent cells using relatively long incubation times for uptake measurements lower affinities of cations are observed compared with 1-second measurements in dissociated cells. In recent measurements, we observed a 6-fold higher K_m value for MPP^+ uptake in human embryonic kidney (HEK)293 cells stably transfected with rat OCT1, when uptake was measured in confluent cells when 2-minute uptake measurements were performed compared with 1-second uptake measurements in dissociated cells (S. Rahman, V. Gorboulev, C. M. Albert, U. Roth, M. Meyer, M. Tzvetkov, T. D. Mueller, H. Koepsell, unpublished data). Likewise, for MPP^+ uptake by HEK293 cells stably transfected with human OCT1, a K_m value of 79 $\pm$ 17 μM was obtained using 2-minute uptake measurements in confluent cells (see Supplemental Table 1 in Matthaei et al., 2016), whereas we determined a K_m value of 9.95 $\pm$ 0.32 μM for MPP^+ uptake by dissociated CHO cells stably transfected with human OCT1 using 1-second uptake measurements (see Fig. S4C in Minuesa et al., 2009). The different K_m values obtained in the different uptake assays could be the result of three factors. First, in the absence of intracellular substrate, OCT1 operates as an electrogenic cation uniporter, whereas OCT1 operates as electroneutral cation exchanger in the

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ABBREVIATIONS: CHO, Chinese hamster ovary; HEK, human embryonic kidney; MPP^+, *N*-methyl-4-phenylpyridinium; OCT1, organic cation transporter 1; TDF, tenofovir disoproxil fumarate.

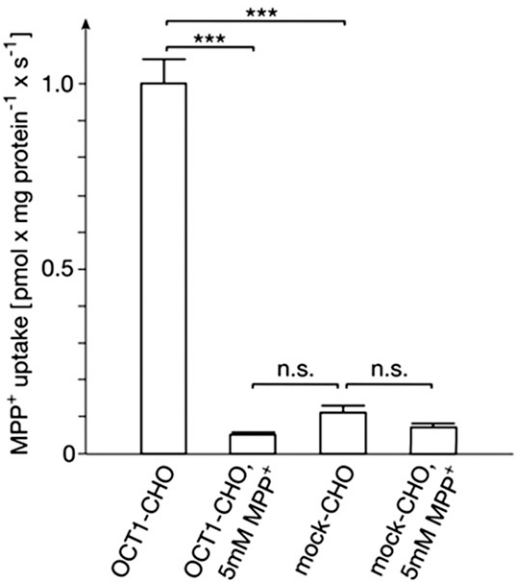

Fig. 1. MPP^+ uptake in CHO cells stably transfected with human OCT1 compared with CHO cells that had been stably transfected with the empty vector pcDNA5. In dissociated cells 1-second uptake of 1 μM radioactively labeled MPP^+ was measured in the absence or presence of 5 mM non-radioactive MPP^+. A representative experiment is shown. Mean values ± SEM of four determinations are indicated. ***$P < 0.001$ tested by ANOVA with posthoc Tukey comparison; n.s., difference is not significant.

presence of a saturating intracellular substrate concentration (Koepsell, 2011). When 1-second uptake measurements are performed, most probably the electrogenic uniport transport mode is determined, whereas the electroneutral antiporter mode may be analyzed when uptake is measured using an incubation time of 1 or 2 minutes. Different transport modes are also suggested by the observation that the rate of MPP^+ uptake within the first seconds is much higher than later on (see Fig. S4A in Minuesa et al., 2009, and Fig. D in the letter of Shen and coworkers). Second, the regulatory state of OCT1, which has an impact on affinities of substrates and inhibitors (Ciarimboli and Schlatter, 2005), may be different in confluent versus dissociated cells, as has been discussed for human OCT2 (Thévenod et al., 2013). Third, during incubation of confluent cells with substrate using gentle shaking, unstirred layer effects may lead to determination of high apparent K_m and IC_{50} values compared with 1-second uptake measurements in dissociated cells performed using vigorous shaking (Minuesa et al., 2009).

The relatively high substrate concentrations used by Shen and coworkers in their uptake measurements (1 μM MPP^+ and 2 μM for metformin) are probably a major reason for the failure to detect inhibition of OCT1 by TDF. Recently we observed that rat OCT1 contains two MPP^+ binding sites per transporter monomer with relatively low dissociation constant K_D values similar to the K_m value of MPP^+ (T. Keller, V. Gorboulev, F. Bernhard, V. Dötsch, H. Koepsell, unpublished data). These binding sites are supposed to be directly involved in cation translocation. In addition, rat OCT1 contains high-affinity cation binding sites that probably modulate cation transport allosterically (Gorbunov et al., 2008; Koepsell, 2015). When transport is measured in the presence of different cation concentrations far below the respective K_m value, different loading of the high-affinity cation binding sites by the substrate may alter the structure of the cation binding region and thereby influence binding and effects of inhibitors. In a previous review, the impact of substrate concentrations on affinities of inhibitors was suggested to explain different inhibitor affinities reported from different laboratories (Nies et al., 2011). As an example, for inhibition of MPP^+ uptake in HEK293 cells transfected with human OCT1, an IC_{50} value of 0.4 μM was reported using a MPP^+ concentration of 0.1 μM and 1-minute incubation of confluent cells for uptake measurements (Jung et al., 2008), whereas in confluent CHO cells transfected with human OCT1, an IC_{50} of 16 μM was determined using 1 μM MPP^+ and 4-minute incubation (Ming et al., 2009). In Fig. 2 we present data showing clear differences in IC_{50} values for inhibition by pentamidine of HEK293 cells transfected with human OCT1 and for inhibition of human OCT2 by quinindine when different MPP^+ concentrations far below the K_m value of MPP^+ were used for the uptake measurements. For inhibition of OCT1-expressed uptake by pentamidine, respective IC_{50} values of 11.1 ± 3.2 μM and 7.8 ± 2.5 μM were obtained when uptake was measured with 0.1 μM or 1 μM MPP^+, whereas an IC_{50} of 0.46 ± 0.2 μM was determined when uptake of 0.1 nM MPP^+ was measured (mean values ± S.D. from three independent experiments, $P < 0.01$ significances for difference of measurements with 0.1 and 1 μM MPP^+ versus measurement with 0.1 nM MPP^+). Measuring the inhibition of OCT2-expressed MPP^+ uptake by quinindine using 1 nM, 0.1 μM, or 1 μM MPP^+ for the uptake measurements, we observed lower IC_{50} values with 0.1 μM and 1 μM MPP^+ (4.6 ± 0.7 μM and 5.6 ± 3.6 μM, respectively) compared with 1 nM MPP^+ (124 ± 20 μM) (mean values ± S.D. from three independent experiments, $P <$ 0.001 significance for difference of measurements with 0.1 and 1 μM MPP^+ versus measurement with 1 nM MPP^+).

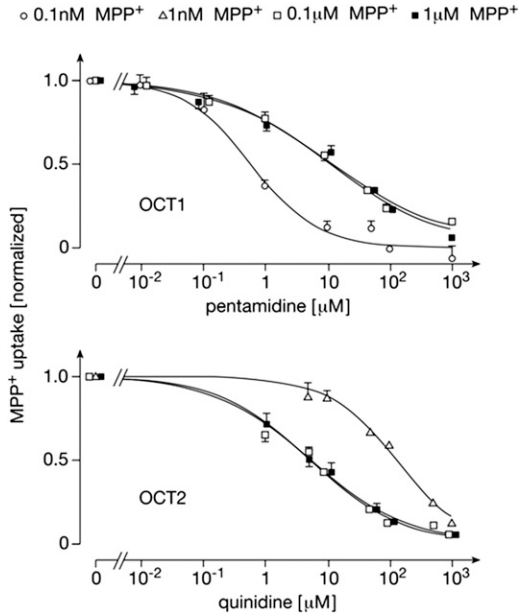

Fig. 2. Inhibition of cation uptake mediated by OCT1 and OCT2 may be inhibited with different affinities when different substrate concentrations far below their K_m values are employed. Inhibition of human OCT1 by different concentrations of pentamidine or of human OCT2 by different concentrations of quinidine was measured in HEK293 cells that had been stably transfected with OCT1 or OCT2. Uptake in the presence of pentamidine or quinidine was measured by incubating dissociated cells for 1 second with MPP^+ concentrations of 0.1 nM, 1 nM, 0.1 μM, or 1 μM. Mean values ± S.E.M. of 12 determinations from three experiments are indicated. The curves were obtained by fitting the Hill equation to the data.

The observation that not only the molecular structure of OCT substrates (Thévenod et al., 2013) but also their concentration may have a dramatic impact on the affinity of inhibitors must be considered for in vitro testing of new drugs for drug-drug interactions (Koepsell, 2015). In fact, rilpivirine, a non-nucleoside reverse transcriptase inhibitor approved for use in treatment-naive adult HIV-infected patients and frequently administered in combination with TDF and emtricitabine (FTC), has shown inhibition of OCT1 in vitro (Moss et al., 2013). Thus, interaction of multiple antiretroviral drugs with OCT1 should always be considered to unveil potential drug-drug interactions. To determine a potential interaction of a new drug with OCT1, OCT2, and/or OCT3 with a drug that is translocated by the respective transporter, inhibition of clinically relevant free concentrations of the transported drug by the new drug has to be investigated.

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