Colistin is substrate of the carnitine/organic cation transporter 2 (OCTN2, SLC22A5)

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Abbreviations: carnitine/organic cation transporter 2, OCTN2; organic cation transporter, OCT1; solute carrier, SLC; tetraethylammonium bromide, TEA.
Abstract

Colistin is a polycation antibiotic used for the treatment of multidrugResistance (MDR) gram-negative infections; nevertheless, its use is often limited by the high incidence of renal damage. The mechanism underlying colistin-induced nephrotoxicity is not known, but perhaps related to its accumulation in the renal cortex upon extensive reabsorption from the nascent urine. Because little is known about the membrane transport of colistin, the purpose of the present study was to characterize better the transport system involved in colistin renal handling by using HEK293 cells stably transfected with the main organic cation transporters expressed at the apical membrane of the proximal tubule. [14C]Colistin was transported by the carnitine/organic cation transporter 2 (OCTN2, SLC22A5) but not by the organic cation transporter 1 (OCT1) and N1 (OCTN1). Non-labeled colistin inhibited the OCTN2-mediated transport of [3H]L-carnitine in a non-competitive manner and that of [14C]tetraethylammonium bromide ([14C]TEA) in a competitive manner. Unlike that of [3H]L-carnitine, the [14C]colistin OCTN2-mediated uptake was Na+-independent. When endogenous OCTN2-mediated colistin transport was inhibited by co-incubation with L-carnitine, primary mouse proximal tubular cells were fully protected from colistin toxicity, suggesting that colistin toxicity occurred upon intracellular accumulation.
Introduction

Colistin, a member of the polymyxin family, represents the mainstay for the treatment of multidrug-resistance (MDR) gram-negative bacterial infections (Falagas and Kasiakou, 2006). The main limitation of colistin treatment is nephrotoxicity (Deryke et al., 2010; Garonzik et al., 2011; Dalfino et al., 2012). The mechanism underlying colistin nephrotoxicity is not known. It has been suggested that colistin acts in a detergent-mode, increasing membrane permeability and, in turn, inducing cell swelling and lysis (Falagas and Kasiakou, 2006). More recently, Yun and colleagues proposed, by using fluorescent-probed polymixins, that the renal damage induced by polymixins is exerted upon entering the cells (Yun et al., 2015).

Colistin enters proximal tubular cells from the apical side upon extensive reabsorption from the nascent urine (Li et al., 2003). Due to its polycationic structure colistin poorly diffuses across the plasma membrane so that it requires facilitative transport systems to enter the cells. Studies in isolated perfused rat kidney investigating the effect of different compounds on the renal disposition of colistin revealed that tetraethylammonium (TEA) and the dipeptide glycine-glycine (Gly-Gly) could increase colistin clearance, indicating that renal reabsorption of colistin may involve organic cation transporters and peptide transporters (Ma et al., 2009). In line with this, the oligopeptide transporter 2 (PEPT2, SLC15A2) was recently shown to transport [³H]polymyxin B1 and the uptake was substantially abolished by co-incubation with non-labeled colistin (Lu et al., 2016).

The present work identified the carnitine/organic cation transporter 2 (OCTN2, SLC22A5) as novel transport system of colistin at the apical side of proximal tubule cells and demonstrated that colistin toxic activity occurred at the intracellular level.
Materials and Methods

Reagents
Radiolabeled colistin [methyl-\(^{14}\text{C}\)]methylated ([\(^{14}\text{C}\)]colistin) was purchased from American Radiolabeled Chemicals (St. Louis, MO). L-[methyl-\(^{3}\text{H}\)]carnitine hydrochloride ([\(^{3}\text{H}\)]L-carnitine) was synthesized by Amersham Life Sciences (Piscataway, NJ). [1-\(^{14}\text{C}\)]-Tetraethylammonium Bromide ([\(^{14}\text{C}\)]TEA) was provided by Perkin Elmer (Boston, MA). Non-labeled colistin, L-carnitine and TEA were provided by Sigma-Aldrich (St. Louis, MO). All cell culture reagents were purchased from Gibco (Parsley, UK).

Cell lines
Wild-type HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO\(_2\). OCT1-HEK293 cells, previously characterized, were supplemented with Geneticin G-418 (600 µg/ml) (Thevenod et al., 2013). OCTN1- and OCTN2-HEK293 cells were maintained in 800 µg/ml Geneticin G-418 (Tamai et al., 1997; Tamai et al., 2001).

Animals
Animal experiments and protocols conformed to the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health) and the Swiss animal protection laws and were approved by the Cantonal Veterinary Office in Zurich, Switzerland (study number 2012058).

Isolation of primary proximal tubular cells from mouse kidney
Primary proximal tubular cells were isolated from kidneys of female C57/BJ mice as described previously (Gai et al., 2010; Gai et al., 2016). Briefly, kidney cortices were dissected, sliced, minced, and digested in 0.25% collagenase solution (Life Technologies BRL, Grand Island, NY). Collagenase was neutralized with Bovine Serum Albumin (BSA). The suspension was filtered, centrifuged (72.4 x g for 5 minutes) to pellet the tubules, washed with 10 ml of medium, centrifuged, and washed twice more. The final pellet, containing of renal tubules, was resuspended in culture medium (REBM bullet kit, Clonetics, Basel, Switzerland), plated onto culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO₂.

**Transport Studies in Intact Cells**

Uptake of radiolabeled compounds was measured using a protocol designed for uptake determination in cells (Schroeder et al., 1998; Visentin et al., 2015). Cells were seeded in 35-mm dishes coated with 0.1 mg/ml poly-D-lysine (Corning, Bedford, MA). Cells were washed and equilibrated in transport buffer (116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH₂PO₄, 0.8 mM MgSO₄, 5.5 mM D-glucose and 20 mM Hepes/Tris, pH 7.4) at 37°C then the buffer was aspirated and transport buffer containing the radiolabelled substrate was added. Uptake was stopped by quick aspiration followed by extensive washing with ice-cold transport buffer. Transport in Na⁺-free buffer was measured as described above, in a transport buffer in which NaCl was replaced with choline chloride (116.4 mM choline chloride, 5.3 mM KCl, 1 mM KH₂PO₄, 0.8 mM MgSO₄, 5.5 mM D-glucose and 20 mM Hepes/Tris, pH 7.4).

Cells were solubilized and intracellular radioactivity was assessed by liquid scintillation counting. Protein content was determined by the bicinchoninic acid protein assay (Interchim, Montluçon Cedex, France). For Dixon’s analysis, OCTN2-independent influx was determined in WT-HEK293 cells and subtracted from total uptake to quantify
the OCTN2-mediated influx. Uptake is expressed as picomoles of substrate per milligram of protein.

To evaluate colistin accumulation as a function of the extracellular concentration of L-carnitine, proximal tubular cells were seeded in 96-well plates at a density of 0.5x10^4 cells/well in a medium containing [14C]colistin at the extracellular concentration of 100 µM. After 48h exposure cells were extensively washed with ice-cold transport buffer and then processed, as described previously, for radioactivity and protein content.

**Viability Assay**

Primary proximal tubular cells were seeded in 96-well plates at a density of 0.5x10^4 cells/well in a medium containing a fixed concentration of colistin (100 µM), and varying the concentration of L-carnitine (0.075, 0.3, 1.2, 5 mM). Viability rates were quantified after 48h of exposure by alamarBlue® assay (Thermo Fisher Scientific, Carlsbad, CA). Fluorescence (λ_ex = 520nm, λ_em = 580nm) was measured on the GloMax Multi Detection System (Promega, Madison, WI).

**Statistical Analysis**

Statistical comparisons were performed from three independent measurements, at least, using GraphPad Prism (version 5.0 for Windows, GraphPad Software).

**Results**

**Impact of OCT1, OCTN1 and OCTN2 expression on the [14C]colistin net uptake in HEK293 cells**

To understand the role of the organic cation transporters expressed at the luminal side of the proximal tubular cells in colistin uptake, HEK293 cells stably transfected with
OCT1 (a), OCTN1 (b) or OCTN2 (c) were incubated at the indicated times with [14C]colistin at an extracellular concentration of 1 µM. As illustrated in figure 1, [14C]colistin was transported only by OCTN2 (Fig. 1c). After 10 min incubation the intracellular level of [14C]colistin in OCTN2-HEK293 was significantly higher than that in WT-HEK293 cells (9.6 ± 1.2 vs 7.2 ± 0.7, P=0.04).

Effect of colistin on OCTN2-mediated transport

The main physiological role of OCTN2 in the kidney is the tubular reabsorption of carnitine (Nezu et al., 1999). OCTN2 can also transport cations such as tetraethyammonium bromide (TEA). Initial uptake of [3H]L-carnitine (Fig. 2a) and [14C]TEA (Fig. 2c) was measured in OCTN2- and WT-HEK293 cells. It can be seen that the uptake slopes of [3H]L-carnitine and [14C]TEA was linear over the first 20 seconds and 3 minutes, respectively, reflecting the unidirectional flux (influx) of the radiolabeled compounds into these cells. To study whether colistin could inhibit the OCTN2-mediated transport of [3H]L-carnitine and [14C]TEA, the inhibitory effect of non-labeled colistin on the influx of [3H]L-carnitine and [14C]TEA was measured. To further study the nature of the inhibition, the influx of [3H]L-carnitine or [14C]TEA at three different concentrations of substrate as a function of extracellular non-labeled colistin at the indicated concentrations was measured (Dixon analysis). In figure 2b it can be seen that the lines intersect on the x-axis (-Ki), indicating that colistin could inhibit the OCTN2-mediated [3H]L-carnitine transport in a non- or un-competitive manner (Ki = 22.7 ± 0.9 mM), suggesting that colistin and [3H]L-carnitine did not bind to a common binding site. Figure 2d indicates that the nature of the inhibition of [14C]TEA by colistin was competitive and the Ki value could be estimated to be ~ 2.5 mM.
Effect of Na⁺ on the colistin uptake mediated by OCTN2

OCTN2 mediates the uptake of the zwitteronic substrate L-carnitine in a Na⁺-dependent manner, whereas the transport of TEA is Na⁺-independent (Tamai, 2013). To investigate whether colistin OCTN2-mediated transport was driven by Na⁺, the uptake of [¹⁴C]colistin at an extracellular concentration of 1 µM was assessed in the presence or absence of Na⁺. Figure 3 shows that [¹⁴C]colistin uptake mediated by OCTN2 was similar in the presence and absence of Na⁺ in the transport buffer.

Contribution of OCTN2 to colistin accumulation and toxicity in primary proximal tubular cells

To understand the contribution of endogenous OCTN2 to colistin cellular uptake and toxicity, the accumulation and toxic effect of colistin in mouse renal primary proximal tubular cells was assessed by co-incubation with different extracellular concentrations of L-carnitine (0.075, 0.3, 1.2, 5 mM). Figure 4 shows that, at an extracellular concentration of 100 µM, colistin alone reduced cell viability by ~ 25%. The intracellular accumulation (H₀, slope=0, P=0.005) and the toxic effect (H₀, slope=0, P=0.01) of colistin significantly decreased as the extracellular concentration of L-carnitine increased. Cells exposed to the highest extracellular concentration of L-carnitine (5 mM) showed 100% viability and coincided with ~ 30% less intracellular [¹⁴C]colistin, in line with the Kᵢ value of colistin for the OCTN2-mediated L-carnitine transport.

Discussion

Among the organic cation transporters expressed at the luminal side of the proximal tubule, the carnitine/organic cation transporter 2 (OCTN2, SLC22A5) could transport colistin at a pharmacologically relevant concentration (1 µM), suggesting that
this carrier might contribute to the renal handling of colistin in the clinical setting (C_{\text{max}}, 1-10 \, \mu \text{M}) (Couet et al., 2011; Karvanen et al., 2013). The model for OCTN2-mediated transport envisages two distinct but overlapping binding sites for carnitine and cationic compounds (Ohashi et al., 2002). OCTN2 transports zwitterionic carnitine in a Na\(^+\)-dependent high-affinity manner, whereas organic cation transport (e.g. Tetraethylammonium bromide (TEA)) is a low-affinity, Na\(^+\)-independent process (Tamai, 2013). Colistin is likely to interact with the cationic binding site of the pocket, as suggested by the competitive inhibition of non-labeled colistin with [\(^{14}\)C]TEA at the OCTN2 level. TEA co-administration could reduce colistin reabsorption in isolated perfused rat kidney. The authors speculated that OCTN1 mediates, at least in part, colistin renal reabsorption (Ma et al., 2009). Our data indicate that OCTN2 and not OCTN1 can transport colistin and that such transport is Na\(^+\)-independent.

Previously colistin was shown to inhibit the transport mediated by PEPT2 at clinically relevant concentrations. The same investigators, in the process of screening different carriers for colistin transport, could not observe any significant inhibition of OCTN2-mediated L-carnitine transport when co-incubated with colistin. This was probably due to the relatively low concentration of colistin (10 \, \mu \text{M}) used for the cis-inhibition assay, as indicated by the relatively high K_i of colistin for [\(^3\)H]L-carnitine transport reported in the present study (Lu et al., 2016). Cis-inhibition data from experiments with primary cultured mouse proximal tubular cells suggests that OCTN2 gives a relevant contribution to colistin reabsorption, in line with the work from Ma and colleagues in isolated perfused rat kidney (Ma et al., 2009).

The physiological role of OCTN2, at the kidney level, is to mediate the reabsorption of carnitine from the glomerular filtrate (Tamai et al., 1998; Tamai et al., 2001). Drugs that alter the expression and/or activity of OCTN2 have been associated with carnitine deficiency (Heuberger et al., 1998; Ganapathy et al., 2000; Diao et al., 2001).
2010; Lancaster et al., 2010). When the ratio \( \frac{C_{\text{max}}}{K_i} \) is considered to forecast the possibility of a clinical drug-drug interaction (>0.1), based upon the relatively low \( C_{\text{max}} \) achievable with the current recommended dosage (1-10 µM) and the high \( K_i \) for carnitine transport calculated (~ 22 mM), colistin-induced carnitine deficiency is unlikely (Greenblatt, 2009; Couet et al., 2011; Karvanen et al., 2013). Nevertheless, rats treated with a single intraperitoneal administration of colistin showed no sign of renal damage, but a 2-4 fold increase in the levels of carnitine in urine (Jeong et al., 2016). The drug-drug interaction observed in the animal study might be due to the much higher \( C_{\text{max}} \) reached (50-100 µM) as result of the high dose administered (Hengzhuang et al., 2012; Jeong et al., 2016).

The molecular mechanism underlying colistin-induced nephrotoxicity is unknown. The detergent property of the d-aminobutyric acid and fatty acid components may damage the plasma membrane of the cells. This hypothesis, however, does not account for the selective toxicity of colistin towards proximal tubular cells (Falagas and Kasiakou, 2006; Ordooei Javan et al., 2015). Alternatively, nephrotoxicity may be secondary to the intracellular accumulation of colistin (Yun et al., 2015). By co-incubation of primary cultured proximal tubular cells with \([^{14}\text{C}]\)colistin and L-carnitine we provide direct evidence that higher intracellular accumulation of colistin resulted in a more potent toxic effect. While L-carnitine is a high affinity substrate for OCTN2, its inhibitory effect on \([^{14}\text{C}]\)colistin uptake is relatively weak, as the result of the noncompetitive/uncompetitive nature of the inhibition.

Although the colistin detergent-mode activity cannot be completely ruled out, especially at suprapharmacological concentrations of the drug, the results support the hypothesis that colistin toxicity occurs upon intracellular accumulation. Since OCTN2 is abundantly expressed at the luminal side of the kidney proximal tubule, the current
findings provide one possible explanation for the particular sensitivity of the kidney parenchyma to colistin exposure (Tamai et al., 1998; Tamai et al., 2001).
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Authorship contributions
Participated in research design: Michele Visentin, Zhibo Gai and Gerd A. Kullak-Ublick.

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Reference List


Footnotes

Conflict of Interest: The authors have no conflict of interest to declare.

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Figure Legends

**Figure 1. Time course of the net uptake of [14C]colistin in transfected cells.**
Uptake of [14C]colistin at the extracellular concentration of 1 µM in OCT1- (a), OCTN1-(b), OCTN2-HEK293 cells (c) was measured as the function of the time and compared with that in wild-type cells. Data represent the mean ± S.E.M. from three independent experiments.

**Figure 2. Effect of non-labeled colistin on OCTN2-mediated transport.** Initial uptake of [3H]L-carnitine (a) and [14C]TEA (c) at the extracellular concentration of 0.1 µM and 50 µM, respectively, in OCTN2- and WT-HEK293 cells. [3H]L-carnitine (15 seconds) (b) and [14C]TEA (2 minutes) (d) influx at the indicated extracellular concentrations as a function of increasing extracellular concentrations of non-labeled colistin was measured in OCTN2 transfected cells. Uptake in WT-HEK293 cells was subtracted from that in transfected cells to define the OCTN2-specific transport. The line was derived by plotting 1/v against the concentration of colistin (inhibitor) (Dixon analysis).

**Figure 3. OCTN2-mediated transport in Na⁺-free buffer.** OCTN2-mediated uptake of [3H]L-carnitine and [14C]colistin at the extracellular concentration of 1 µM. Uptake in WT-HEK293 cells was subtracted from that in transfected cells to define the OCTN2-specific transport. Results are expressed as percentage of the uptake values in transport buffer containing Na⁺. Data represent the mean ± S.E.M. from three independent experiments.
Figure 4. Colistin accumulation and cell survival as a function of L-carnitine extracellular concentration in mouse primary proximal tubular cells. Cells were co-exposed to [14C]colistin at the extracellular concentration of 100 µM and non-labeled L-carnitine at the indicated concentrations. After 48h exposure, survival rate and intracellular radioactivity were measured. Left and right y-axes indicate respectively the intracellular [14C]colistin and the cell survival, as the function of the extracellular concentration of L-carnitine. Data are expressed as percentage of the respective controls and represents the mean ± S.E.M. from 4 independent experiments.
Figure 1

(a) Intraocular $[^{14}C]$colistin pmol/mg of protein vs. time (min) for OCT1 and WT.

(b) Intraocular $[^{14}C]$colistin pmol/mg of protein vs. time (min) for OCTN1 and WT.

(c) Intraocular $[^{14}C]$colistin pmol/mg of protein vs. time (min) for OCTN2 and WT.
Figure 2

(a) OCTN2

(b) [\text{L-carnitine}] = 0.1 \mu M, 1 \mu M, 10 \mu M

(c) OCTN2

(d) [\text{TEA}] = 50 \mu M, 250 \mu M, 500 \mu M

Intracellular [\text{L-carnitine}] pmol/mg of protein

Intracellular [\text{TEA}] pmol/mg of protein

Time (sec)

Time (min)

[colistin] mM

[colistin] mM
Figure 3

Intracellular substrate % of control

$[^3]H$L-carnitine

$[^4]C$colistin

+ NaCl

+ Choline Chloride
Figure 4

The graph illustrates the percentage of control for intracellular $[^{14}C] \text{colistin}$ and survival against the logarithm of the concentration of $\text{L-carnitine}$ in the range of 2 to 4 log units. The data is represented by circles for uptake and squares for survival.