Renal Disposition of Colistin in the Isolated Perfused Rat Kidney

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Nephrotoxicity is an important limitation to the clinical use of colistin against Pseudomonas aeruginosa and other gram-negative pathogens. Previous work reported net tubular reabsorption of colistin by the kidney in vivo, but there is no knowledge of its disposition within the kidney. This study investigated the renal disposition and potential transport mechanisms of colistin in the isolated perfused rat kidney (IPK) model by perfusing with colistin sulfate alone (2 μg/ml) or in the presence of potential inhibitors (tetraethylammonium [TEA], glycine-glycine [Gly-Gly], or hydrochloric acid [HCl]) at three different concentrations. When perfused alone, the renal clearances (CLR) for colistin A and B (the major components of colistin) in control kidneys were constant and low (mean values ≤ 0.05 ml/min throughout the perfusion). The mean clearance ratios [CR, defined as CLR(fu) × GFR, where ffu is the fraction of drug unbound in perfusate and GFR is the glomerular filtration rate] were significantly less than 1. It was concluded that there is net tubular reabsorption of colistin, and this exceeded the reabsorption of water. Less than 10% eliminated from perfusate was recovered in urine, suggesting considerable renal accumulation of colistin. The CR values for colistin were significantly increased when perfused with TEA (500 μM, Gly-Gly (833 μM), and HCl (2,500, 5,000, and 10,000 μM). It is proposed that renal reabsorption of colistin may involve organic cation transporters (inhibited by TEA) and peptide transporters (inhibited by Gly-Gly) and that the process is sensitive to the pH of urine.

Infections caused by gram-negative bacteria, in particular, Pseudomonas aeruginosa, Acinetobacter baumannii, and Klebsiella pneumoniae, are emerging as global health issues (26). Increasingly, infections caused by many strains of these bacteria are very difficult to treat because of resistance to most of the currently available antibiotics (16). Furthermore, there is a very worrying shortage of new antibacterials under development for the treatment of infections caused by gram-negative bacteria (17, 26).

Colistin (polymyxin E, Fig. 1) is an important member of the polymyxin class of cationic polypeptide antibiotics, with the major components being colistin A (polymyxin E1) and colistin B (polymyxin E2). It is administered to humans as colistin methanesulfonate (CMS), an inactive prodrug that requires B (polymyxin E2). It is administered to humans as colistin methanesulfonate (CMS), an inactive prodrug that requires

As opposed to the “high” incidence of nephrotoxicity with the early clinical use of intravenous CMS (6), it is now recognized that the incidence of nephrotoxicity is not as high as was previously thought, but there is no doubt that the administration of CMS has the potential to cause kidney damage (7, 15). Furthermore, the toxicity of CMS is remarkably lower than colistin (1), and it is generally accepted that nephrotoxicity arising from the administration of CMS is related to the colistin, formed in vivo, that is delivered to the kidney (15). Clearly, the potential for colistin to cause nephrotoxicity may be linked to the way in which it is handled by the kidney.

A previous study found a very low renal clearance of colistin after intravenous administration of colistin (as its sulfate salt) in rats, consistent with extensive renal tubular reabsorption (14). However, nothing is known of the mechanisms involved in the renal tubular transport of colistin. The rat isolated perfused kidney (IPK) model is ideal for examining the renal disposition, including tubular cell transport mechanisms, of endogenous compounds and drugs (2). Thus, the present study was designed to investigate the renal disposition and transport mechanisms of colistin in the rat IPK by perfusing colistin, in the absence or presence of several potential renal transport inhibitors.

MATERIALS AND METHODS

Materials. Colistin sulfate, polymyxin B sulfate, [3H]inulin, bovine serum albumin (fraction V), dextran (molecular weight, 64,000 to 76,000), tetraethylammonium (TEA), glycine-glycine (Gly-Gly), L-cysteine, glycine, L-glutamic acid and mannitol were purchased from Sigma-Aldrich (St. Louis, MO). D-Glucose of Analar grade was purchased from Ajax Chemicals (Auburn, NSW, Australia). Cellulose nitrate membrane filters were purchased from Sartorius (Goettingen, Germany); aqueous counting scintillant from Amersham (Arlington Heights, IL); Centricon Centrifugal Filter Devices (YM-10; molecular weight cutoff, 10,000) from Millipore Corp. (Bedford, MA); and Multiple Reagent Strips for Urinalysis from Bayer Diagnostics Business Group (Pymble, NSW, Australia). Hydrochloric acid (HCl) of Analar grade was purchased from BDH Laboratory Supplies (Poole, United Kingdom). Water was purified by using a Milli-Q system (Bedford, MA).

The isolated perfused rat kidney preparation and experimental design. The study was approved by the Animal Ethics Committee of the Institute of Medical
and Veterinary Science (IMVS). Male Sprague-Dawley rats (400 to 450 g) from the IMVS were maintained at ~25°C on a 12-h light/dark cycle with free access to food and water.

The IPK preparation was based on a previously published method (30). An equilibration of 20 min was allowed after placing the kidney in the thermostatic cabinet before adding [3H]inulin (130 kBq) into the perfusate reservoir. After 5 min, the perfusate volume in the recirculating system was adjusted to 160 ml by the addition or removal of perfusate. For each perfusion, colistin sulfate stock solution (1 mg/ml, 0.32 ml) was added into the reservoir as a bolus to achieve an initial concentration of 2 µg/ml (equivalent to ~1.27 µM summed colistin A and B); this time was defined as 0 min.

A total of 20 perfusions were performed, divided into four experimental groups (n = 5 per group). Each group was perfused with colistin in the absence or presence of incrementally escalating concentrations of TEA, Gly-Gly, or HCl. Each perfusion was divided into period I (5 to 30 min), period II (35 to 55 min), period III (60 to 80 min), and period IV (85 to 105 min). The stock solution of TEA, Gly-Gly, or HCl was added into the reservoir as a bolus at 30, 55, and 80 min to achieve low, medium, and high concentrations, respectively (Table 1). A 5-min equilibration was allowed after the addition of colistin or the inhibitors; urine was then collected over 5-min intervals within each period, and perfusate samples (0.6 ml) were collected from the reservoir at the midpoint of each interval. Urine volume was measured gravimetrically in preweighed collection vials and urine flow rate (UFR) was calculated accordingly. Immediately after completion of the perfusion, aliquots of the perfusate (100 µl) or urine (50 µl) samples were added to scintillation vials and mixed with 3 ml of aqueous counting scintillator, and the levels of radioactivity were counted by using a liquid scintillation analyzer (model 2200CA; Packard, Canberra, Australia). The remaining samples were stored at ~20°C pending analysis for colistin.

Binding in perfusate. For determination of the unbound fraction (fu) of colistin in perfusate, samples were prepared containing colistin sulfate at 1 and 2 µg/ml in perfusate and allowed to equilibrate at 37°C for 30 min. Quadruplicate samples (2 ml) at each concentration were dispensed into the chamber above the membrane of Centricon tubes and capped, and the tubes were centrifuged at 25°C on a 12-h light/dark cycle with free access to food and water.

The product of CLu and GFR represents the clearance of (unbound) colistin by the rate of filtration at the glomerulus. The CR values within each period were used as the indicator of whether there was net reabsorption (in which case CR would be < 1) or net secretion (in which case CR would be > 1) of colistin.

The averages of values for each 5-min interval were calculated within each group. Averages of values for each period within each group were presented as mean ± the standard deviation (SD). The parameters for periods II, III, and IV in each group were compared to both the values for period I within the same group as well as the values in the corresponding period of the control group using analysis of variance, with a Dunnett’s test used for post hoc comparison. One-sample and paired student t tests were used as appropriate.

### RESULTS

The parameters reflecting viability of the IPKs within each period, as assessed by the UFR, GFR, and %TRwater, are presented in Fig. 2. No time-dependent changes in these parameters were observed in the control group (P > 0.80), and for most periods in the inhibitor treatment groups were not significantly changed (P > 0.80) compared to period I for the respective group. However, the GFR and %TRwater were significantly decreased (P < 0.05) in period IV for the HCl group

### TABLE 1. IPK study design for adding the potential renal transport inhibitors

<table>
<thead>
<tr>
<th>Treatment group (n = 5)</th>
<th>Inhibitor concn (µM)</th>
<th>Period I (5–30 min)</th>
<th>Period II (35–55 min)</th>
<th>Period III (60–80 min)</th>
<th>Period IV (85–105 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>TEA</td>
<td>0</td>
<td>5</td>
<td>50</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Gly-Gly</td>
<td>0</td>
<td>8</td>
<td>83</td>
<td>833</td>
<td></td>
</tr>
<tr>
<td>HCl</td>
<td>0</td>
<td>2,500</td>
<td>5,000</td>
<td>10,000</td>
<td></td>
</tr>
</tbody>
</table>

fu = Colistin sulfate was added as a bolus at 0 min to reach an initial concentration of 2 µg/ml for each perfusion. The potential inhibitors were added at 30, 55, and 80 min to reach low, medium, and high initial concentrations, respectively.
FIG. 2. Kidney viability parameters—UFR (a), GFR (b), and %TR water (c)—of the IPKs. The data are presented as the mean ± the SD (n = 5). * P < 0.05 compared to the value for the control period (period I) in the same group and with the corresponding period in the control group.
In all groups except the HCl group, the perfusate pH was between 7.40 and 7.60 throughout and the urine pH was \(~6.4\), and there were no period-dependent variations observed. For the HCl group, the mean (± the SD) values for perfusate pH in periods I, II, III, and IV were 7.49 ± 0.05, 7.18 ± 0.03, 6.86 ± 0.11, and 5.02 ± 0.65, respectively, and the corresponding values for urinary pH were 6.4, 6.2, 5.9, and 4.9. The perfusate and urinary pH of period II, III, and IV in the HCl group were significantly decreased from the value in period I of the same group (\(P < 0.05\)).

The concentrations of colistin A and B in perfusate at the end of the perfusion were around half of their initial values (Fig. 3). Less than 10% of the decrease in the amount in perfusate was finally recovered in urine. For colistin A, the \(f_{tu}\) at 1 \(\mu\)g/ml and 2 \(\mu\)g/ml was 0.41 and 0.43, respectively, while the value for colistin B at both concentrations was 0.60. The values for \(f_{tu}\) of colistin A and B at both concentrations were not significantly different in the presence of the high concentrations of the inhibitors (Table 1) included in the present study (\(P > 0.78\)). Thus, mean values of 0.42 and 0.60 for colistins A and B, respectively, were used for calculation of the CR.

In the control group, mean values for CL\(_{R}\) of colistin during each period were in the range from 0.028 to 0.040 ml/min, and there was no period-dependent variance (\(P > 0.92\)) in the values (Fig. 4). Also, the mean CL\(_{R}\) of colistin B was slightly higher than the corresponding value of colistin A, but the difference was not significant (\(P > 0.21\), paired Student \(t\) test), whereas the values of CR for colistin A and B were almost identical (\(P > 0.43\), paired Student \(t\) test). Within each period of this group, the CR values were significantly less than unity in all periods (Fig. 5, \(P < 0.01\), one-sample \(t\) test). Mean values for the %TR\(_{colistin}\) were significantly higher (\(P < 0.05\), paired Student \(t\) test) than the corresponding values for %TR\(_{water}\).

There were no significant (\(P > 0.36\)) differences in kidney viability parameters, or in the CL\(_{R}\) or CR of colistin between period I of each treatment group and the corresponding values of the control group. Again, %TR\(_{colistin}\) was also significantly higher (\(P < 0.05\), paired student \(t\) test) than the corresponding %TR\(_{water}\). However, both CL\(_{R}\) and CR for colistin were significantly (\(P < 0.05\)) increased by the high concentration of TEA (500 \(\mu\)M) and Gly-Gly (833 \(\mu\)M) (i.e., in period IV), but not by the low and medium concentrations of these potential inhibitors (Fig. 4 and 5). When HCl was added to perfusate to achieve concentrations of 2,500, 5,000, and 10,000 \(\mu\)M in periods II, III, and IV, respectively, the CL\(_{R}\) and CR for colistin were significantly (\(P < 0.05\)) increased compared to period I but it was significantly less than for period III (Fig. 4, \(P < 0.05\)). The CR of colistin in period III of the HCl group was further increased compared to period III (\(P < 0.05\) and was greater than unity for both colistin A and B (\(P < 0.05\) in the final period (Fig. 5). The CR values of colistin A in the treatment period, when renal reabsorption of colistin was significantly inhibited, were substantially higher than the corresponding value of colistin B (paired Student \(t\) test, \(P < 0.05\)).

**DISCUSSION**

The fact that the CR in the above-mentioned experimental periods was <0.1 (see Fig. 5) indicates that >90% of the
Colistin in tubular urine underwent reabsorption. Extensive reabsorption of colistin in the IPK is in agreement with the earlier study in vivo in rats (14). The finding that the %TR\textsubscript{colistin} of both colistin A and colistin B in the control group (>90%) was significantly higher than the corresponding %TR\textsubscript{water} (~80%, Fig. 2c) indicates that the tubular reabsorption of colistin must have been, at least in part, via a carrier-mediated mechanism; the same conclusion was reached from studies conducted in vivo in rats (14). The excellent agreement between the renal disposition observed in vivo and that in the IPK model supports the use of the latter model for investigating the mechanisms involved in the renal handling of colistin.

Protein binding of colistin in IPK perfusate was studied by ultrafiltration using concentrations of colistin sulfate approximating the initial and end values measured during the perfusions. The $f_u$ values (0.42 for colistin A and 0.60 for colistin B) were similar to but slightly higher than the values for rat plasma reported by Li et al. (0.36 for colistin A and 0.52 for colistin B) (14), most likely because there was less protein in the perfusate.

A study in our laboratory indicated that colistin is very stable in perfusate at 37°C, with more than 90% remaining for up to 24 h. Therefore, accumulation within renal cells would most probably contribute to the low urinary recovery (<10%) of colistin eliminated from perfusate in the IPK study. Polymyxins have been found to be bound extensively and persistently to a range of organs after parenteral administration (5, 10, 11, 33).

FIG. 4. Mean (± the SD) CL\textsubscript{r} for colistins A and B during each period in each group. The concentration of inhibitor in each period is given in Table 1. *, $P < 0.05$ compared to the values of period I in the same group and to the values in the same period of the control group.
and tended to be eliminated very slowly from these tissues (33). Clearly, the carrier-mediated reabsorption observed and the postulated accumulation in the kidney may have implications for renal toxicity.

To study the renal transport of colistin, we focused on potential membrane transporters known to be responsible for the transport of substrates from the lumen back into tubular cells and which might account for the extensive net reabsorption observed for colistin in the current study and in vivo (14). The existence of five \(\gamma\)-amine groups (Fig. 1) with an estimated pKa of \(~10\) means that colistin is a polycation under physiological conditions in both perfusate (pH \(~7.4\)) and urine (pH \(~6.4\)). Thus, it may be transported into tubular cells by the organic cation transporters, such as OCTN1 and OCTN2, on the brush-border membrane of tubular cells in the kidney (8). The latter functions mainly as a \(\text{Na}^+\)/carnitine antiporter with a high affinity for carnitine, but it may, however, also mediate organic cation uniporter in a \(\text{Na}^+\)-independent manner (8). On the other hand, OCTN1 is a multispecific, bidirectional, \(\text{Na}^+\)-independent and pH-dependent cation transporter located mainly in the kidney. TEA is a typical substrate for rat OCTN1 (31, 32). It has been proposed that human OCTN1 may represent the luminal \(\text{H}^+\)/cation antiporter which uses the \(\text{H}^+\) gradient generated by the sodium-proton antiporter to translocate intracellular cations across the brush-border membrane (9, 20). On the other hand, colistin features a peptide structure (heptapeptide ring and tripeptide side chain). Thus, we focused also on polypeptide transporters.

![Graph](image)

**FIG. 5.** Mean (± the SD) CR for colistins A and B during each period in each group. The concentration of inhibitor in each period is given in Table 1. *, \(P < 0.05\) compared to the values of period I in the same group and to the values in the same period of the control group.
(PEPT1 and PEPT2) expressed in the renal cells which mediate the reabsorption of oligopeptides (23). In the kidney, PEPT2 is more abundant than PEPT1 and expressed predominantly in the apical membrane of the epithelial cells in the proximal tubule (19). Typical substrates for PEPT include di- or tripeptides, as well as β-lactam antibiotics and cephalosporins (12). Gly-Gly is the simplest dipeptide and a typical substrate/inhibitor for PEPT used in several previous studies (4, 28). Given the dependence of the OCTN1 transporter on pH (31, 32); therefore, its impact was also examined in the present study along with the other two inhibitors.

Renal excretion of colistin did not alter when coperfused with TEA or Gly-Gly at low and medium concentrations, which may suggest a high affinity between colistin and the renal transporters (most probably, OCTN1 and PEPT). The CR of TEA can be estimated to be between 2 and 3 after intravenous administration to dogs and humans (21, 22). Therefore, in the present study, the concentrations of TEA achieved in urine in period IV of the TEA treatment group would be much higher than the \( K_m \) value for TEA (436 µM) observed with hOCTN1-transfected HEK293 cell (27). Thus, the increased \( C_{LR} \) and CR of colistin in period IV may be due to the accelerated efflux of colistin accumulated within tubular cells, where OCTN1 functions as an organic cation/organic cation antiporter (Fig. 6c). Meanwhile, TEA may also competitively inhibit the reabsorption of colistin via OCTN1 (Fig. 6d).

Typical substrates for PEPT include di- or tripeptides, as well as β-lactam antibiotics and cephalosporins (12). There has been no report thus far regarding the renal transport via PEPT of larger molecules such as colistin. However, it is possible that renal reabsorption of colistin, a peptide-based molecule, is occurring via PEPT in the kidney, and this is being inhibited by Gly-Gly, the typical inhibitor/substrate of this transporter. On the other hand, Gly-Gly has a net negative charge at physiological pH (Sigma-Aldrich Gly-Gly [G1002] product information sheet). Therefore, in addition to competitive inhibition by Gly-Gly of the reabsorption of colistin via PEPT, the availability of colistin for reabsorption via OCTN1 could be reduced by the formation of an ion-pair between Gly-Gly and colistin within the tubular lumen (Fig. 6e). Previous workers proposed that an ion-pair interaction between an organic cation (TEA) and an organic anionic drug (cefdinir) reduced the tubular reabsorption of cefdinir (13).

As mentioned previously, the pKa of colistin is \(~10\); thus, the pH of perfusate and urine in each period would not substantially change its ionic status, with most of the drug remaining positively charged. The \( C_{LR} \) and CR of colistin in the treatment periods of the HCl group were increased to the greatest extent compared to other inhibitor groups, which suggested that the renal transport of colistin is highly pH sensitive. In the HCl group, the perfusate pHs during periods II and III were slightly and moderately acidic, respectively, whereas the renal function remained unchanged, as shown by the kidney viability parameters. However, the GFR and \( %T_{R\text{water}} \) in period IV of the HCl group were significantly decreased (\( P < 0.05 \)) compared to period I and proteinuria was observed, which together suggested that there was underlying pathology associated with severe acidosis. During periods I to III for this group, inhibition of the renal reabsorption of colistin (both colistins A and B) was associated with decreasing pH of the perfusate. The potential mechanisms most likely involve the bidirectional transport and pH-dependent properties of OCTN1. Efflux into urine of colistin which accumulated within tubular cells during period I was assumed to be accelerated by increased proton concentration in luminal urine in period II, III

**FIG. 6.** Schematics of possible mechanisms for the renal tubular transport of colistin. (a) Tubular reabsorption of colistin, where OCTN1 functions as a proton/organic cation antiporter. (b) Increased proton gradient may accelerate the efflux of colistin from renal tubular cells. (c) TEA increases the efflux of colistin, where OCTN1 functions as an organic cation/organic cation antiporter. (d) TEA competitively inhibits the reabsorption of colistin via OCTN1, where OCTN1 functions as a proton/organic cation antiporter. (e) Gly-Gly may form a complex with colistin by forming ion pairs, which may disrupt the reabsorption of colistin via OCTN1.
and IV (Fig. 6b); efflux during period IV may be a combination of this plus pathological damage to tubular cells.

The human homologs of OCTN1 and PEPT have tissue distributions, membrane localizations, transport properties, and substrate specificities very similar to those of the rat (12, 24, 25, 29, 31). Thus, it would be speculated that colistin may be transported in very similar manner in human kidney, as observed in the IPK in the present study.

In conclusion, this is the first study examining the renal disposition of colistin in the IPK and to explore its possible mechanisms of tubular transport. Colistin was rapidly eliminated from the perfusate, but only a relatively low fraction of colistin and a considerable amount of colistin that was reabsorbed in the kidney tissue. Such cellular accumulation may have implications for its well-established renal toxicity. The tubular reabsorption of colistin was inhibited by TEA, Gly-Gly, and HCl. It was concluded that the reabsorption of colistin is most likely mediated by OCTN1 but it may also occur via PEPT.

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