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SHORT COMMUNICATION

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Reversible nephrotoxicity of onconase and effect of lysine pH on renal onconase uptake

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Abstract *Purpose*: To examine the histopathology of the kidney in mice following repeated injections of the antitumor drug onconase, and to determine whether lysine, which reportedly blocks kidney uptake of other basic proteins, blocks the high renal uptake of onconase. *Methods*: Mice received repeated intraperitoneal onconase injections over 3 weeks. Kidneys were examined by light microscopy after 1 week, 3 weeks, and 5 weeks (2 weeks after cessation of injections) and compared to kidneys from animals receiving a similar schedule of PBS injections. Renal uptake of radioiodinated onconase was measured in animals receiving intraperitoneal injections of lysine solutions of acidic and neutral pH given at −30, 0 and +5 min relative to intravenous onconase injection. Renal onconase uptake was also measured in animals made metabolically acidotic by ingestion of ammonium chloride, arginine chloride or lysine dihydrochloride from the drinking water. *Results*: Onconase caused acute moderate multifocal proximal renal tubular necrosis, and this toxicity was reversed by 2 weeks after drug withdrawal. Intraperitoneal injections of lysine dihydrochloride in PBS (pH 1.5) reduced renal onconase uptake at 15 min from 67.9 ± 13.4% to 17.0 ± 3.8% of the injected dose without affecting the plasma concentration and also reduced the fraction of degraded onconase in the urine. However, neutral solutions of lysine dihydrochloride at pH 7.4 or lysine acetate at pH 7.1 were ineffective at blocking renal onconase uptake. Furthermore, renal onconase uptake was minimally or not affected by a state of metabolic acidosis. *Conclusions*: Proximal tubular toxicity of onconase was reversible. Renal onconase uptake was blocked by lysine at pH 1.5 but not at neutral pH.

Key words Ribonuclease · Nephrotoxicity · Lysine · pH · Metabolic acidosis

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Introduction

Extracellular ribonucleases, bovine seminal ribonuclease, bullfrog lectin, and onconase, are capable of restricting the growth of tumors [5, 10, 11, 18, 23]. Onconase, present in the eggs and embryos of the common leopard frog *Rana pipens*, is cytotoxic to numerous tumor cell lines and also inhibits the proliferation of intraperitoneal (i.p.) implanted M109 Madison mouse carcinoma cells in Balb/c mice [5, 14, 23]. It is presently undergoing clinical evaluation for treatment of mesothelioma. In addition to an antiproliferative effect, onconase displays antiviral activity and inhibits the replication of HIV-1 virus in infected H9 leukemia cells [24].

Phase I clinical trials have shown that nephrotoxicity, characterized by proteinuria with edema and variable azotemia, is the dose-limiting toxicity of onconase [15]. Preclinical studies in rats and dogs have shown primarily liver toxicity [15]. On the other hand, pharmacokinetic and biodistribution studies in mice have shown that, in this species, the majority of injected onconase is taken up by the kidneys, and unlike other closely related

ribonucleases, is removed from the kidney very slowly [21]. The mouse therefore appeared to be a good animal model to further investigate the renal toxicity of onconase and to seek a method for blocking renal uptake which might allow dose intensification to improve the efficacy of onconase.

Solutes such as amino acids and low molecular weight proteins easily pass through the kidney glomerular capillary wall into the filtrate and are mostly reabsorbed by the epithelial cells of the proximal tubule [12]. Lysine and other basic amino acids reportedly inhibit absorption of several basic proteins [17], presumably by competing with positively charged amino groups of the protein for binding to receptors on the surface of proximal tubular cells. Systemic administration of lysine has been reported to block the kidney uptake of peptides and low molecular weight proteins such as somatostatin analogs and $F(ab')_2$, Fab or Fv fragments of monoclonal antibodies [3, 6–8, 19]. The aim of the present study was to examine the toxic effect of onconase on the kidneys of mice and to determine whether lysine could inhibit renal uptake of onconase.

Materials and methods

Materials

Onconase, purified from the eggs of *Rana pipens* according to the procedure of Ardelt et al. [1], was a gift from Alfacell (Bloomfield, N.J.). Its molecular weight is 11 800 Da, and its isoelectric point is greater than 9.4 [21]. Bolton-Hunter reagent was purchased from New England Nuclear, Boston, Mass. Lysine dihydrochloride and lysine acetate were obtained from Sigma Chemical Co., St. Louis, Mo.

Radioiodination of onconase

Onconase was iodinated by a modification of the Bolton-Hunter procedure as described previously [21] and chromatographed and dialyzed prior to use. The final specific activity was 4 mCi/mg and >95% of the radioactivity was precipitable by 12.5% (w/v) trichloroacetic acid (TCA) using bovine serum albumin (BSA) as carrier.

Animals

Normal female mice (3–4 weeks old) of the Balb/c strain (NCI, Frederick, Md.) were used for the studies. Animals were acclimated to the housing conditions for a week and were kept on water containing 0.01% NaI during this period. The animals were treated according to study protocols approved by the NIH Animal Care and Use Committee. The mice weighed 18 ± 2 g at the time of the experiment.

Dosing protocol and histopathology

Four mice in each of three groups were injected i.p. with 8 μg onconase (~0.44 mg/kg body weight or 1.3 mg/m^2) in 0.2 ml phosphate buffered saline (PBS) daily for five consecutive days in a week. Mice were not injected on days 6 and 7 of that week. The total weekly dose of onconase was 40 μg/mouse. The first group was sacrificed after 5 days of therapy, and the second group after 3 weeks (19 days) of therapy. Mice in the third group received the same drug regimen as the second group and then were kept off drug therapy for an additional 2 weeks. Control mice were injected with the same volume of PBS on the same schedule as the treated groups. The mice were weighed every second day. The mice were humanely sacrificed, urine was tested for protein (N-Multistix SG, Bayer Corp., Tarrytown, N.J.), and the kidneys and livers were immediately removed. The organs were then fixed in 10% neutral buffered formalin, embedded in paraffin, routinely sectioned, stained with hematoxylin and eosin, and examined by light microscopy by a veterinary pathologist (J.A.B.). Lesions were graded on a scale of severe, moderate, mild, or no damage.

Amino acid preparations

Stock solutions of 230 m*M* L-lysine dihydrochloride and L-lysine acetate were prepared in PBS pH 7.4. The final pH of the solutions were 1.5 and 7.1, respectively. For preparation of L-lysine dihydrochloride solution of pH 7.4, the pH of a stock solution of 460 m*M* in 0.1 *M* phosphate buffer pH 7.4 was adjusted with NaOH, and then diluted with the same buffer to 230 m*M*.

Biodistribution experiments

An intravenous (i.v.) injection of 10 μg radioiodinated onconase was administered via the tail vein. Three doses of 0.2 ml each of 230 m*M* lysine solutions were administered i.p. at −30 min, 0 and +5 min relative to i.v. onconase injection (~7.7 mmol lysine/kg body weight or 23 mmol/m^2). PBS pH 7.4 was injected into control animals. Time-points studied were 15 and 120 min. At the indicated times, a sample of blood was obtained from the tail vein. This was used for hematocrit determination as well as for measuring the amount of radioactivity as described in previous study [21]. The mice were sacrificed, urine was collected from the urinary bladder using a syringe, and kidneys and liver were excised, weighed and counted for radioactivity. From the total uptake by the tissues, the radioactivity contributed by the plasma space was subtracted as described previously [21], and data were expressed as percent of injected dose (ID). Urine radioactivity was determined before and after precipitation in 12.5% TCA. The two-tailed Student's *t*-test assuming unequal variances was used to test for statistical significance between the control and lysine-treated animals.

Metabolic acidosis studies

To induce a state of metabolic acidosis, a 0.28 *M* solution of ammonium chloride, arginine hydrochloride or lysine dihydrochloride containing 100 m*M* sucrose was substituted for the drinking water of the mice. Ingestion of ammonium chloride in the drinking water had been previously shown to lower plasma pH in rats to 7.28 and urine pH to 5.6 [22]. Control animals received 100 m*M* sucrose or 0.28 *M* lysine acetate in 100 m*M* sucrose. Urine pH was measured prior to switching the water and at various times thereafter with pH test strips. Maximal pH depression occurred between one and two days. In subsequent experiments, animals received one of the metabolic acidosis-inducing solutes in the drinking water for 16 h. Urine pH was measured, and the animals received an i.v. injection of 10 μg radiolabeled onconase. A biodistribution experiment and statistical analysis were carried out as described above.

Results and discussion

Histopathology

A phase I clinical trial of onconase in cancer patients has shown that the organ of dose-limiting toxicity is the kidney. Clinical symptoms are proteinuria with edema and, occasionally, azotemia [15]. Biodistribution studies

in mice have shown that i.v. administered onconase is taken up primarily by the kidneys (60–70% ID at 15 min), and ~50% ID is retained even after 180 min [21]. Prolonged tissue retention is also observed in liver, although onconase uptake by the liver is only about 1% ID. Therefore, for histopathological studies, we examined only the kidney and liver. One group of mice received five daily i.p. injections of onconase. Another group received a 3-week course of therapy (five daily i.p. injections/2 days no injection). A final group of mice was sacrificed 2 weeks after ending 3 weeks of therapy. Within a week of therapy, onconase-treated mice showed clinical signs of toxicity such as ruffled coat and lethargy. Over the 3-week period, a 3–4 g decline in body weight was observed in these mice. Control mice appeared normal with a slight weight loss of 0.5 g. Urinary protein concentrations in both the control and treated mice were similarly low, measuring +1 on a scale of 0 to +4, or approximately 30 mg/dl.

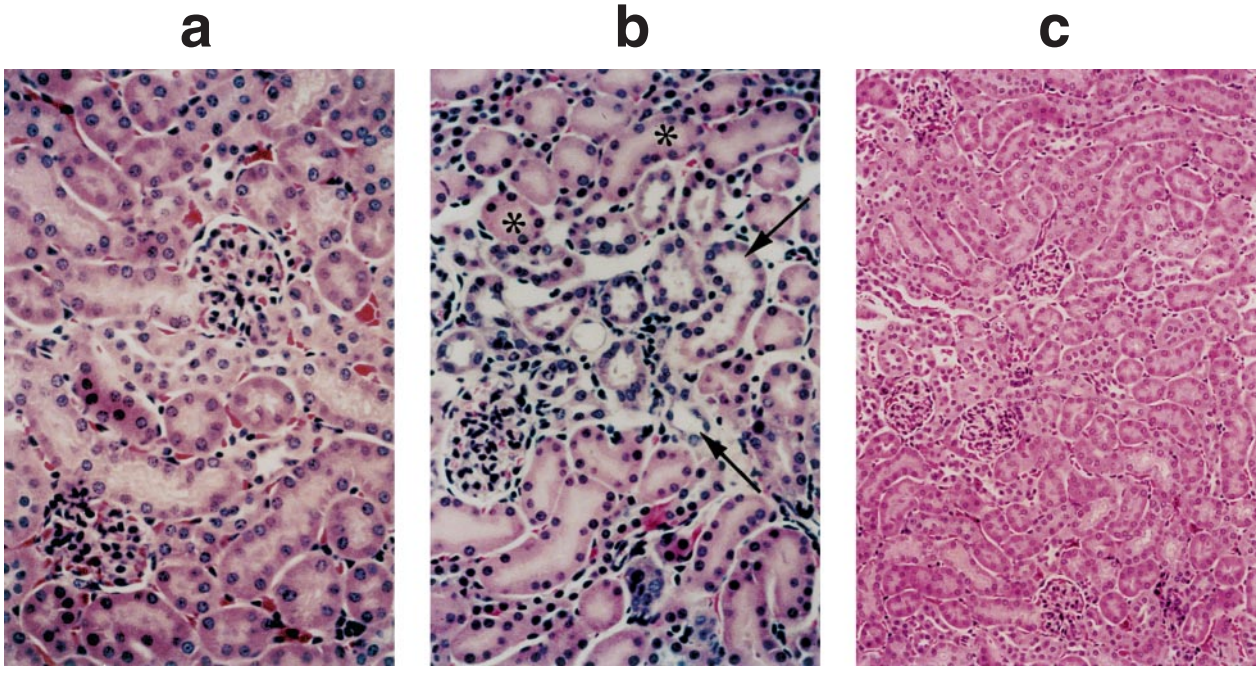
Histopathological examination of the kidneys revealed that the microscopic nephrotoxic effect of onconase was limited to the proximal tubules and was reversible. In mice after 3 weeks of onconase therapy, the kidneys showed multifocal moderate acute necrosis of the proximal convoluted tubules (Fig. 1b). Other structures in the kidney such as distal tubules, glomeruli, medulla, pelvices and blood vessels appeared unaffected. Many proximal tubules were dilated and lined by fewer cells, indicating loss by necrosis of epithelial cells. Some epithelial cells in these tubules were necrotic or degenerative, and a few were regenerative. The toxic effect on the kidneys was essentially similar after 1 week of therapy except that the number of affected tubules was roughly one-half as many as at 3 weeks (data not shown). Kidney sections from control animals injected with buffer showed no necrosis, dilated tubules or regeneration (Fig. 1a). Therefore, there appeared to be a distinct toxic effect of onconase on the renal proximal tubules. Kidneys from mice 2 weeks after conclusion of 3 weeks of onconase therapy showed no tubular damage (Fig. 1c). There were scattered regenerative tubules, indicative of past damage. The normal appearance indicates that the histologically detected toxic effects of onconase were reversed after the drug was withdrawn.

Microscopically in the liver, toxic effects were seen in the hepatocytes. Lipidosis, cellular swelling, vacuolar degeneration and apoptosis were observed in mice treated for 1 and 3 weeks. Following the 2-week recovery period, the liver appeared normal, indicating that the microscopic hepatotoxicity was also reversible.

Effects of amino acids and metabolic acidosis on renal handling of onconase

The toxic effect of onconase on the proximal tubules prompted investigations to prevent accumulation of onconase in the kidneys. Low molecular weight proteins of the molecular size of onconase are readily filtered by the glomerular membrane, and these are mostly reabsorbed and metabolized by the epithelial cells lining the wall of the proximal tubules [12]. Mogensen and Solling

Fig. 1a–c Photomicrographs of hematoxylin and eosin-stained kidneys. **a** Normal renal cortex from a control animal (400×). **b** Focal nature of proximal tubular damage after 3 weeks of onconase injections. *Arrows* indicate region of tubular dilatation, degeneration and necrosis; *asterisks* indicate nearby normal tubules (400×). **c** Normal renal cortex in a mouse given 3 weeks of drug treatment and then allowed to recover for 2 weeks (200×)



have shown that i.v. administered dibasic amino acids such as lysine, arginine, and ornithine block reabsorption of proteins by the proximal tubules, and this blockage increases excretion of β2-microglobulin, albumin and free light chains of immunoglobulins [17]. Systemic administration of lysine has also been used to block the kidney uptake of the indium-labeled somatostatin analog, pentetreotide, thereby improving the sensitivity of this reagent in detection of gastroenteropancreatic tumors [7]. Radioconjugated antibody fragments have also been used extensively as tumor diagnostic reagents but their high renal accumulation can lead to nephrotoxicity in addition to compromising their ability to detect tumors. Lysine is effective in decreasing the kidney uptake of these radiolabeled conjugates [3, 6, 8, 19]. We therefore decided to study the effect of coadministration of lysine on the accumulation of onconase by the kidneys.

To block the renal uptake of i.v. injected indium-labeled Fab fragment of monoclonal antibody in mice, Pimm and Gribben administered lysine dihydrochloride i.p. at −1, 0, +1, +2, +3 h with respect to the radiolabeled antibody [19]. We initially experimented with a similar dose schedule, administering lysine dihydrochloride at −1, 0, and +1 h relative to onconase and sacrificing animals at 2 h, or administering lysine dihydrochloride at −1 and 0 h and sacrificing animals after 15 min. No blockage of renal onconase uptake was found with this schedule. Kobayashi et al. then reported that the timing of the lysine dose within minutes of an i.v. injection of Fv fragment is crucial to successful blockade of renal accumulation of Fv fragment [8]. We found that i.p. injected lysine dihydrochloride given −30, 0, +5 min with respect to i.v. administered iodinated onconase achieved a good inhibition of renal onconase accumulation. This schedule was used in subsequent experiments.

Following injection of radiolabeled onconase, the plasma concentrations in animals receiving lysine dihydrochloride (pH 1.5) and PBS were very similar at 15 min: 11.0 ± 7.6% and 10.9 ± 6.2% ID/g, respectively (mean ± SD). This indicates that the plasma clearance of onconase was not affected by the lysine injections. The amount of radioactivity in the kidneys, however, was much lower in the lysine-treated animals: 17.0 ± 3.8% ID compared to 67.9 ± 13.4% ID in control animals at 15 min (Table 1).

Macromolecules in the glomerular filtrate are reabsorbed and degraded into amino acids in the tubular portion of the kidney. The extent of onconase degradation was assessed by measuring the percentage of urine radioactivity that could be precipitated by TCA. At 15 min, the percentage was 11.8 ± 6.1%. In other words, most of the radioactivity was degraded protein or free iodine. When onconase was injected with lysine dihydrochloride, the precipitable radioactivity increased to 89.9 ± 11.0% (Table 1). This suggests that lysine coadministration substantially prevented onconase degradation.

At 120 min, almost all of the injected onconase was cleared from the plasma in both groups of animals, with a slightly, though not significantly ($P > 0.05$), higher plasma concentration in the lysine dihydrochloride-treated animals: 1.7 ± 0.4% and 0.7 ± 0.1% ID/g in lysine-treated and control animals, respectively. In the control animals, onconase persisted in the kidneys with 48.6 ± 5.3% ID still remaining. By contrast, only 9.3 ± 0.5% ID was found in the kidneys of the lysine dihydrochloride-group. Degradation in the urine showed a similar result to that at 15 min. In the PBS-treated group, only 1.8 ± 0.4% of the urine radioactivity was precipitable, whereas 90.2 ± 2.5% was precipitable in the lysine dihydrochloride-treated group. Liver uptake of onconase was not affected by lysine dihydrochloride at 15 min or 120 min: 1.0 to 1.3% ID was in the liver regardless of coinjection of lysine (Table 1).

Having demonstrated that lysine dihydrochloride blocked renal uptake of onconase, we planned to evaluate the histopathology of kidneys from mice given repeated injections of onconase coadministered with lysine. In preparation for the experiment, we gave a

Table 1 Effect of lysine pH on onconase uptake and degradation. Values are means ± SD with the number of samples shown in parentheses (*ND* not done)

	Plasma (% ID/g)	Kidney (% ID)	Liver (% ID)	Percent acid precipitable in urine
15 min				
PBS (pH 7.4)	11.0 ± 7.6 (9)	67.9 ± 13.4 (9)	1.1 ± 0.4 (7)	11.8 ± 6.1 (6)
Lysine dihydrochloride (pH 1.5)	10.9 ± 6.2 (10)	17.0 ± 3.8* (11)	1.0 ± 0.3 (7)	89.9 ± 11.0* (7)
Lysine dihydrochloride (pH 7.4)	5.0 ± 0.5 (4)	67.2 ± 3.8 (4)	ND	46.0 ± 15.1* (3)
Lysine acetate (pH 7.1)	7.7 ± 0.5 (4)	56.7 ± 9.9 (4)	ND	29.9 ± 3.2* (4)
120 min				
PBS (pH 7.4)	0.7 ± 0.1 (3)	48.6 ± 5.3 (3)	1.1 ± 0.4 (3)	1.8 ± 0.4 (3)
Lysine dihydrochloride (pH 1.5)	1.7 ± 0.4 (3)	9.3 ± 0.5* (3)	1.3 ± 0.1 (3)	90.2 ± 2.5* (3)

* $P < 0.02$ vs PBS group, two-tailed Student's *t*-test with unequal variances

group of mice daily i.p. injections of 230 m*M* lysine dihydrochloride (three injections of 0.2 ml each) and unexpectedly found that the animals became moribund after 3 days. Discovering that the pH of this solution was very low (1.5), we prepared solutions of 230 m*M* lysine dihydrochloride at physiological pH 7.4 and gave repeated daily i.p. injections (three injections of 0.2 ml each) for up to 3 weeks. These were well-tolerated and produced no microscopic renal toxicity. We therefore tested the ability of neutral lysine dihydrochloride to prevent the renal uptake of onconase. Coadministration of lysine dihydrochloride at pH 7.4 or lysine acetate at pH 7.1 did not lower renal onconase uptake compared to that in control animals (Table 1). The neutral solutions of lysine were associated with higher levels of undegraded onconase in the urine compared to the controls, but were not nearly as effective as lysine dihydrochloride at pH 1.5. Therefore, the pH of the coadministered lysine solution appears to be an important factor affecting reabsorption of onconase by kidney proximal tubules.

Surmising that the pH environment in the proximal tubules might play an important role in preventing onconase absorption, we induced metabolic acidosis by adding ammonium chloride, arginine hydrochloride or lysine dihydrochloride to the drinking water. Urine pH gradually decreased, and after 16 h, the pH was depressed to 5.9 (Table 2). Lysine dihydrochloride in the drinking water resulted in lower renal onconase uptake to a small, but significant, degree. Renal onconase uptake in the ammonium chloride and arginine chloride acidotic animals was similar to that of normal controls. Moreover, the percent of undegraded onconase in the urine was similar in all groups of acidotic animals compared to normal controls. Thus, a lowered urine pH resulting from gradually induced metabolic acidosis did not appear to be an effective strategy for substantially altering renal handling of onconase.

Studies reporting the prevention of proximal tubular absorption of other proteins by basic amino acid solutions have been carried out at neutral and acidic pH. An i.p. injection in mice of 0.5 *M* L-lysine monohydrochloride solution with pH adjusted to 7.5 (dose 17–33 mmol lysine/kg body weight, 50–100 mmol/m^2) blocks renin reabsorption by proximal tubules [13]. In human subjects, a 4-h i.v. infusion of Synthamin 14 (pH ~6, 34 m*M* lysine, 100 m*M* arginine, dose ~3.8 mmol/kg body weight, 140 mmol/m^2) decreases the uptake of the indium-labeled somatostatin analog pentetreotide by 66% [7]. In monkeys, a bolus i.v. dose followed by a 30-min continuous i.v. infusion of AminoSyn II 15% (pH 5.8, 108 m*M* lysine, 88 m*M* arginine, dose ~1.1 mmol/kg body weight, 13.2 mmol/m^2), significantly lowers the renal uptake of ^{18}F-labeled anti-Tac Fv antibody fragment. In other studies showing an effect of lysine on the excretion of basic proteins, the final pH of the lysine solutions was not reported or could not be independently determined [2, 6, 8, 17, 19, 20]. Therefore, with the exception of the renin study [13], it is unclear whether the critical variable affecting renal handling of these other proteins is acid load of the coadministered solution, as we have found, or the lysine contained therein.

Methods for lowering the high renal uptake of onconase would allow more aggressive dosing of onconase. Coadministration of lysine dihydrochloride pH 1.5, while effective, was poorly tolerated in the mice and therefore could not be translated to a clinical protocol. Continuous i.v. infusion of slightly acidic solutions such as Synthamin 14 and AminoSyn II are effective in blocking renal uptake of other proteins, but testing their effect on onconase would require larger animals because of the technical difficulties of doing continuous i.v. infusions in mice. Other strategies also merit consideration. Megalin, a glycoprotein (gp330) found extensively on the apical aspect of proximal tubule cells [4], has recently been identified as a quantitatively important receptor for endocytosis of several polybasic drugs [16]. As onconase is a highly cationic protein (pI > 9.4), megalin perhaps also mediates onconase uptake by proximal tubular cells. A 39-kDa receptor associated protein (RAP) binds with high affinity (K_d 8 n*M*) to megalin and interferes with binding of ligands to megalin [9]. Hence, it would be interesting to test whether coadministration of RAP could reduce the high renal uptake of onconase. An alternative approach to reduction of kidney accumulation of onconase is modification of amino acid residues by genetic engineering to create variants that are eliminated more readily by the kidney [21].

In conclusion, we have shown in a mouse model that repeated injection of onconase is associated with proximal renal tubular damage, and the toxic effect is reversed by 2 weeks after drug withdrawal. Lysine injected i.p. at

Table 2 Effect of metabolic acidosis on renal onconase uptake. Values are means ± SD with the number of samples shown in parentheses

Solute added to drinking water (100 m*M* sucrose)	Urine pH after 16 h	Onconase in kidney (% ID at 15 min)	Percent acid precipitable in urine
None	6.41 ± 0.22 (4)	77.8 ± 6.4 (4)	3.9 ± 0.5 (4)
Ammonium chloride	5.89 ± 0.08* (4)	75.8 ± 11.7 (4)	13.7 ± 9.6 (4)
Arginine chloride	5.89 ± 0.12* (4)	74.7 ± 4.1 (4)	8.3 ± 4.6 (4)
Lysine dihydrochloride	5.89 ± 0.26* (4)	64.4 ± 4.2* (4)	4.6 ± 1.5 (4)
Lysine acetate	6.83 ± 0.45 (4)	67.6 ± 9.1 (3)	5.2 ± 3.8 (3)

* $P \leq 0.02$ vs control group, two-tailed Student's *t*-test

very low pH, but not at neutral pH, inhibits the uptake of onconase by the kidneys.

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Note added in proof: Additional studies with i.p. administered solutions of 230 mM glycine, glutamic acid and tartaric acid in PBS further support the substantial effect of pH on renal handling of onconase. Renal onconase uptake at 15 min was measured in similar biodistribution experiments, with the following results: glycine (pH adjusted to 1.6 with HCl) – 27.2% ± 5.3%; glycine (pH 7.4) – 63.4% ± 2.8%; L-glutamic acid hydrochloride (pH 2.2) – 28.3% ± 4.6%; L-glutamic acid monoammonium salt (pH 7.2) – 44.5% ± 5.5%; and tartaric acid (pH 2.8) – 17.8 ± 4.0%. Low pH solutions also significantly increased the TCA precipitable radioactivity in the urine (> 80% precipitable).