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Arginine Residues Are More Effective than Lysine Residues in Eliciting the Cellular Uptake of Onconase

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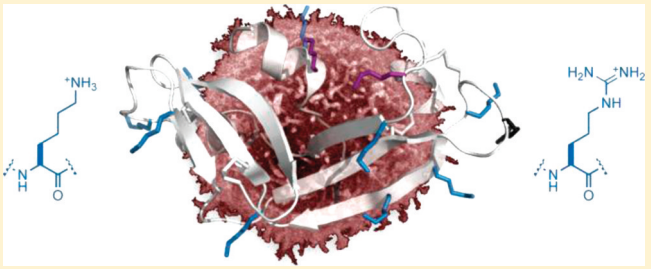
ABSTRACT: Onconase is an amphibian member of the pancreatic ribonuclease family of enzymes that is in clinical trials for the treatment of cancer. Onconase, which has an abundance of lysine residues, is internalized by cancer cells through endocytosis in a mechanism similar to that of cell-penetrating peptides. Here, we compare the effect of lysine versus arginine residues on the biochemical attributes necessary for Onconase to elicit its cytotoxic activity. In the variant R-Onconase, 10 of the 12 lysine residues in Onconase are replaced with arginine, leaving only the two active-site lysines intact. Cytometric assays quantifying internalization showed a 3-fold increase in the internalization of R-Onconase compared with Onconase. R-Onconase also showed greater affinity for heparin and a 2-fold increase in ribonucleolytic activity. Nonetheless, arginine substitution endowed only a slight increase in toxicity toward human cancer cells. Analysis of denaturation induced with guanidine-HCl showed that R-Onconase has less conformational stability than does the wild-type enzyme; moreover, R-Onconase is more susceptible to proteolytic degradation. These data indicate that arginine residues are more effective than lysine in eliciting cellular internalization but can compromise other aspects of protein structure and function.



Delivering an exogenous protein to the cytosol is a challenge. Although some proteins can enter cells by receptor-mediated endocytosis, most do not have cell-surface receptors. Here, we investigate a key biochemical feature of a protein that lacks a cell-surface receptor but has an intrinsic ability to gain entry to the cytosol of human cells.

Pancreatic-type ribonucleases readily undergo endocytosis and can exhibit specific toxicity toward cancerous cells by degrading cellular RNA. Their binding to the cell surface is not saturable[1] and is mediated by nonspecific Coulombic interactions.[2] Onconase (ONC) is an 11.8 kDa pancreatic-type ribonuclease discovered originally in the oocytes of the *Rana pipiens* frog. ONC possesses endogenous antitumoral activity and has been granted fast-track and orphan-drug status for the treatment of malignant mesothelioma.[3−7] Although ONC contains endogenous cytotoxicity, its rate of internalization seems to limit its effectiveness, as the cellular uptake of ONC is only slightly faster than fluid-phase uptake.[8] Moreover, the clinical utility of ONC has been limited by its rapid accumulation in the kidneys,[9,10] resulting in dose-limiting renal toxicity.[11] Although two saturable binding sites with K_d values of 0.062 and 0.25 μM were reported to exist on 9L glioma cells,[12] binding to HeLa cells and CHO cells was shown subsequently to be nonsaturable at concentrations up to 10 μM, indicative of nonspecific binding.[1,13]

Natural cell-penetrating peptides, such as HIV-TAT or penetratin, have a preponderance of cationic residues and are endocytosed readily.[14,15] Interestingly, lysine and arginine residues affect the internalization of such peptides to differing degrees. For example, replacing all of the cationic residues in penetratin with either lysine or arginine revealed a 10-fold advantage for the all-arginine variant compared with the all-lysine variant.[16] This trend is also apparent with synthetic cell-penetrating peptides, as nonaarginine is internalized more readily and is more disruptive to synthetic lipid bilayers than is nonalysine.[17] Arginine forms more stable hydrogen bonds with phosphoryl groups at physiological temperature[18] and could also form a stable interaction with the carboxyl, phosphoryl, and sulfuryl groups on the cell surface, thus facilitating endocytosis.[19]

Increasing the net positive charge (Z) of a protein by either chemical modification or site-directed mutagenesis can increase its internalization.[20−22] For example, green fluorescent protein (GFP) is a highly anionic protein ($Z = -9$) that is not taken up by cells. Replacing five acidic residues with arginine on one face of the folded protein endowed GFP with the ability to undergo endocytosis.[23] Installing even more cationic residues generated a variant ($Z = +36$) that not only undergoes endocytosis but also internalizes pendant cargo.[24] Likewise, installing arginine residues in two homologues, bovine pancreatic ribonuclease (RNase A) and the bullfrog sialic acid-binding lectin, increases their internalization.[25,26] Cytotoxicity has been linked to the net positive charge of a protein[2] as well as the distribution of that charge.[27] None of these studies, however, examined whether lysine and arginine residues have a differential effect on the cellular internalization of a protein and its subsequent function.

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ONC is a highly cationic protein. Among its 104 residues, 12 are lysine and three are arginine (Figure 1[28]), leading to $Z = +5$ and pI > 9.[29] Herein, we report on the consequences of replacing the 10 non-active-site lysine residues of ONC with arginine. We find that the increased arginine content has significant effects on cellular uptake as well as on important biochemical attributes of ONC.

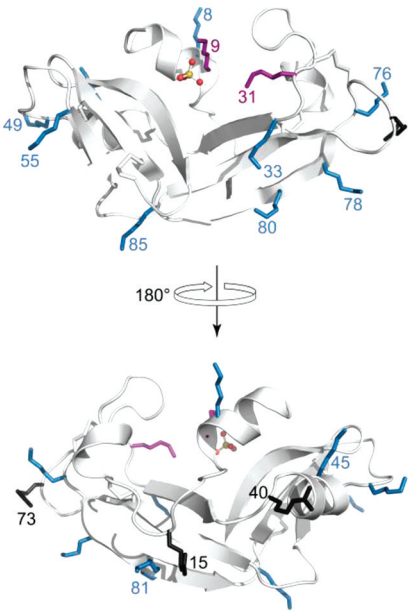


Figure 1. Three-dimensional structure of Onconase (PDB entry code 1ONC[28]). Native arginine side chains are in black; lysine side chains replaced with arginine in R-ONC are in blue; Lys9 and Lys31 are in magenta and are essential for catalytic activity.

EXPERIMENTAL PROCEDURES

Materials. K-562 cells were derived from a continuous human chronic myelogenous leukemia cell line and obtained from American Type Culture Collection (Manassas, VA). *Escherichia coli* strain BL21(DE3) cells and plasmid pET22b(+) were from Novagen (Madison, WI).

Restriction enzymes were from New England Biolabs (Ipswich, MA), and other enzymes were from Promega (Madison, WI) or Sigma–Aldrich (St. Louis, MO). 6-Carboxyfluorescein–dArUdGdA–6-carboxytetramethylrhodamine (6-FAM–dArUdGdA–6-TAMRA) was obtained from Integrated DNA Technologies (Coralville, IA). Cell culture medium and supplements were from Invitrogen (Carlsbad, CA). [*methyl*-^{3}H]Thymidine (6.7 Ci/mmol) was from Perkin-Elmer (Boston, MA). Mes was from Sigma-Aldrich; Mes buffers were purified by anion-exchange chromatography to remove oligo(vinylsulfonic acid), which is a potent inhibitor of ribonucleases that contaminates commercial chemicals derived from ethanesulfonic acid.[30] All other chemicals used were of commercial grade or better and were used without further purification.

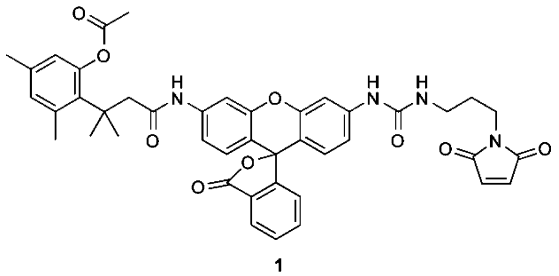
Analytical Instruments. Molecular mass was measured by MALDI-TOF mass spectrometry using a Voyager-DE-PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA). Fluorescence was quantified with an Infinite M1000 plate reader (Tecan, Männedorf, Switzerland). Radioactivity was quantified by scintillation counting using a Microbeta TriLux liquid scintillation counter (Perkin-Elmer, Wellesley, MA). Flow cytometry data were collected in the University of Wisconsin Paul P. Carbone Comprehensive Cancer Center with a FACSCalibur flow cytometer equipped with a 488 nm argon ion laser (Becton Dickinson, Franklin Lakes, NJ).

Production of Ribonucleases. Synthetic DNA encoding a pelB leader sequence followed by R-ONC, in which all lysine residues had been converted to arginine with the exception of the active-site lysines, Lys9 and Lys31, was from GENEART (Burlington, Ontario, Canada). This gene was inserted into plasmid pET(22b)+ using *Nde*1 and *Sal*1-HF restriction enzymes. R-ONC was purified from inclusion bodies as described previously[31] with the following exceptions. R-ONC was folded at 4 °C for 5 days following a slow, 10-fold dilution into 0.10 M Tris-HCl buffer at pH 7.8 containing L-arginine (0.5 M), reduced glutathione (3.0 mM), and oxidized glutathione (0.6 mM). Following concentration by ultrafiltration, R-ONC was purified by FPLC using a Superdex G-75 gel-filtration column (GE Healthcare, Piscataway, NJ) in 50 mM sodium acetate buffer at pH 5.0 containing NaCl (0.10 M) and NaN_3 (0.02% w/v). Further purification by FPLC using a 5 mL HiTrap SPHP column and a linear gradient of NaCl (0.15–0.45 M) in 50 mM sodium acetate buffer at pH 5.0. Protein concentration was determined by ultraviolet spectroscopy using extinction coefficients of $\varepsilon_{280} = 0.87$ (mg mL^{-1})$^{-1}$ cm^{-1} for both ONC and R-ONC.

Production of Labeled Ribonucleases. The purification of R-ONC was complicated by incomplete cleavage of the pelB leader sequence. Accordingly, site-directed mutagenesis was used to replace the pelB leader sequence with a methionine residue to create Met(−1)R-ONC. In addition, Ser61 was replaced with a cysteine residue to create S61C ONC[27] and S61C Met(−1) ONC, which are poised for S-alkylation. Following gel-filtration chromatography, the sulfhydryl groups of S61C Met(−1)R-ONC and S61C ONC were protected by reaction with 5,5′-dithio-bis(2-nitrobenzoic acid), and the resulting proteins were dialyzed overnight against 50 mM sodium acetate buffer at pH 5.0. Protected S61C ONC and S61C Met(−1)R-ONC were purified by FLPC using a 5 mL HiTrap SPHP column and a linear gradient of NaCl (0.15–0.45 M) in 50 mM sodium acetate buffer at pH 5.0. To remove the methionine at its N-terminus, S61C Met(−1)R-ONC was incubated with aminopeptidase for 24 h at 37 °C in PBS at pH 8.0.

S61C variants protected as a mixed disulfide with 2-nitro-5-thiobenzoic acid were deprotected with a 5-fold molar excess of dithiothreitol and then desalted by using a PD-10 desalting column (GE Biosciences, Piscataway, NJ). Deprotected proteins in PBS were reacted for 4–6 h at 25 °C with a 10-fold molar excess of fluorogenic label **1**,[32] which was a generous gift from L. D. Lavis (Janelia Farm Research Campus, Howard Hughes Medical Institute). The reaction was quenched by rapid dilution into 50 mM sodium acetate buffer at pH 5.0. Conjugates were purified by FPLC using a 5 mL HiTrap SPHP column, and the concentration of conjugates was determined with a bicinchoninic acid assay kit (Thermo Fisher Scientific, Rockford, IL) and bovine serum albumin as a standard.

Assays of Catalytic Activity. The ribonucleolytic activity of ONC and its variants was measured by monitoring cleavage of a hypersensitive fluorogenic substrate, 6-FAM–dArUdGdA–6-TAMRA, as described previously.[33] Fluorescence emission at 515 nm, following excitation at 492 nm, in the presence of enzyme was measured in 0.10 M Mes–NaOH buffer at pH 6.0 containing 0.10 M NaCl, 6-FAM–dArUdGdA–6-TAMRA



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(50 nM), and human ribonuclease inhibitor (0.1 nM) at 25 °C. Values for k_{cat}/K_M were calculated with the equation

$$k_{cat}/K_M = \left(\frac{\Delta F/\Delta t}{F_{max} - F_0}\right)\frac{1}{[E]} \quad (1)$$

In eq 1, $\Delta F/\Delta t$ is the initial slope of the reaction, F_0 is the initial fluorescence intensity, F_{max} is the fluorescence intensity once the reaction is brought to completion, and [E] is the concentration of the enzyme.

Cytotoxicity Assays. The effect of ONC and R-ONC on the proliferation of K-562 cells was assayed as described previously.[31] K-562 cells were incubated for 44 h with ribonuclease before treatment with [*methyl*-^{3}H]thymidine for 4 h, at which time the incorporation of radioactive thymidine into cellular DNA was quantitated by liquid scintillation counting. The results are shown as the percentage of [*methyl*-^{3}H]thymidine incorporated relative to untreated cells. Values for IC_{50} were calculated by fitting the curves by nonlinear regression to the equation

$$y = \frac{100\%}{1 + 10^{(\log(IC_{50}) - \log[\text{ribonuclease}])h}} \quad (2)$$

where y is the total DNA synthesis following the [*methyl*-^{3}H]-thymidine pulse and h is the slope of the curve.

Flow Cytometry Assays. The internalization of fluorogenic ONC and R-ONC into K-562 cells was determined by monitoring the deprotection of the fluorophore after encountering intracellular esterases.[27] K-562 cells from near confluent flasks were collected by centrifugation and resuspended at a density of 1×10^6 cells/mL in fresh RPMI 1640 containing FBS (10% v/v). Labeled or unlabeled ribonuclease (10 μM) were incubated with K-562 cells for 3 h at 37 °C. To quench internalization, K-562 cells were collected by centrifugation at 1000 rpm for 5 min at 4 °C, washed once with ice-cold PBS, and resuspended in 300 μL of PBS. Samples remained on ice until analyzed by flow cytometry.

Fluorescence was detected through a 530/30 nm band-pass filter. Cell viability was determined by staining with propidium iodide, which was detected through a 660 nm long-pass filter. The mean channel fluorescence intensity of 10^4 viable cells was determined for each sample with CellQuest software and used for subsequent analysis.

Heparin-Affinity Chromatography. The affinity of ONC and R-ONC for heparin was measured *in vitro*. Each ribonuclease was dissolved in PBS at pH 7.0, and loaded onto a 1.0 mL HiTrap Heparin HP column (GE Healthcare). The column was washed with PBS, and ribonucleases were eluted with a linear gradient of NaCl (0.00–0.45 M) in PBS. Protein elution was monitored by absorbance at 280 nm.

Guanidine-HCl-Induced Transition Curves. The guanidine-HCl-induced unfolding of ONC and R-ONC were monitored by fluorescence spectroscopy. Assays were performed in 100 mM sodium acetate buffer at pH 5.5 containing protein (25 μg/mL) and guanidine-HCl (0–6.8 M). After equilibration, five fluorescence spectra were recorded from 310 to 380 nm. The bandwidth was 5 nm for excitation at 295 and 10 nm for emission. From these spectra, the shift of the wavelength of maximal emission (y) as a concentration of guanidine-HCl was determined and evaluated by nonlinear regression with the program GraphPad Prism 5.0 and the equation[34]

$$y = \frac{y_N^0 + m_N[D] + (y_D^0 + m_D[D])\cdot \exp(-m_{\Delta G}([D]_{50\%} - [D])/RT)}{1 + \exp(-m_{\Delta G}([D]_{50\%} - [D])/RT)} \quad (3)$$

where $[D]_{50\%}$ is the concentration of denaturant [D] at which 50% of the protein is denatured, $m_{\Delta G}$ is the measure of the dependence of the standard free energy on denaturant concentration, y_N^0 and y_D^0 are the intercepts, and m_N and m_D the slopes in the pre- and post-transition region, respectively, in the y versus [D] graph. The fraction of protein in the folded state (f_N) was determined with the equation

$$f_N = (y_D - y)/(y_D - y_N) \quad (4)$$

with

$$y_D = y_D^0 + m_D[D] \quad (5)$$

and

$$y_N = y_N^0 + m_N[D] \quad (6)$$

where y_N and y_D are the signals of the native and denatured protein as a function of denaturant concentration. Values of the standard free energy were calculated with the equation

$$\Delta G^{[D]} = \Delta G^{H_2O} - m_{\Delta G}[D] \quad (7)$$

where ΔG^{H_2O} is the standard free energy of unfolding in the absence of denaturant.

Protease Susceptibility. ONC and R-ONC (0.2 mg/mL) were treated with pepsin at a w/w ratio of 1:2 pepsin/ribonuclease in 50 mM glycine-HCl buffer at pH 2.4 and 37 °C. Aliquots were removed at known times, and aliquots of 2 M Tris were added to raise the pH to 8.0. Samples were analyzed by SDS-PAGE, and bands stained with Coomassie Brilliant Blue R-250[35] were analyzed by densitometry using ImageJ software.

RESULTS

Design, Production, and Purification of R-Onconase. ONC contains three arginine and 12 lysine residues, two of which, Lys9 and Lys31, are necessary for catalytic activity.[33] In the R-ONC variant, all lysines with the exception of Lys9 and Lys31 were replaced with arginine. Thus, R-ONC contains two lysine and 13 arginine residues.

R-ONC was produced in *E. coli* and purified from inclusion bodies. SDS-PAGE and mass spectrometry revealed two distinct protein populations, consistent with the pelB leader sequence not being cleaved from a significant fraction of R-ONC. The N-terminal residue of wild-type ONC is a glutamine, which cyclizes spontaneously to form a pyroglutamate that is critical for catalytic activity.[33] That cyclization requires an N-terminal amino group and thus cannot occur when the pendant pelB leader sequence is intact. R-ONC was separated from pelB-R-ONC

by using an extended gradient during cation-exchange chromatography. This procedure resulted in pure R-ONC (~1 mg/L of culture) as assessed by SDS-PAGE and mass spectrometry (m/z 12 111; expected: 12 100).

Production and Purification of Labeled R-ONC. The conventional production of S61C R-ONC failed because the pelB leader sequence was not cleaved from the protein and because the uncleaved variant was insoluble. Accordingly, site-directed mutagenesis was used to replace the pelB leader sequence with a methionine residue. S61C Met(−1)R-ONC was produced, folded, and purified successfully (m/z 12 322; expected: 12 311). Aminopeptidase catalyzed the hydrolysis of the Met(−1)−Gln1 peptide bond, enabling Gln1 to cyclize into a pyroglutamate residue (m/z 12 147; expected: 12 134). The integrity of labeled R-ONC was verified by MALDI-TOF mass spectrometry (m/z 12 894; expected: 12 873).

Catalytic Activity. The enzymatic activity of a ribonuclease is essential for its cytotoxic activity.[36−38] We found the ribonucleolytic activity of R-ONC (k_{cat}/K_M = (88 ± 17) × 10^3 M^{-1} s^{-1}) to be 2-fold greater than that of ONC (k_{cat}/K_M = (39 ± 9) × 10^3 M^{-1} s^{-1}).

Cellular Internalization. A ribonuclease must enter the cell to exert its cytotoxic activity. We have shown previously that intracellular esterases unmask fluorogenic label **1** within an endosome, enabling quantification using flow cytometry.[13,32] R-ONC exhibited a 3-fold increase in cellular uptake compared to ONC (Figure 2).

Heparin Affinity. Heparan sulfate is known to be important for the internalization of arginine-rich peptides and some pancreatic-type ribonucleases.[13,39] We assessed the relative affinity of R-ONC and ONC for heparin, which is a relatively homogeneous mimic of the GAGs that are abundant components of the mammalian cell surface.[19,40] We found that R-ONC has greater affinity for heparin than does ONC (Figure 3), consistent with its increase in cellular uptake (Figure 2).

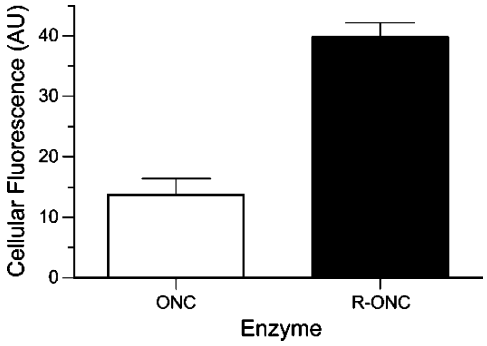

Figure 2. Effect of arginine residues on the cellular uptake of Onconase. Ribonucleases were conjugated via Cys61 to latent fluorophores containing fluorogenic label **1**. Flow cytometry was used to measure the internalization of ONC and R-ONC into K-562 cells. R-ONC displayed increased internalization compared to ONC (p < 0.01).

Cytotoxic Activity. Because of its enhanced ribonucleolytic activity and cellular uptake, R-ONC could be more cytotoxic than ONC. We found, however, that ONC has IC_{50} = 0.4 ± 0.1 μM and R-ONC has IC_{50} = 0.29 ± 0.01 μM (Figure 4), indicative of only a marginal increase in cytotoxicity.

Conformational Stability. Next, we sought a biochemical basis for the unexpectedly low cytotoxic activity of R-ONC. Guanidine-HCl induces the denaturation of proteins,[41] and we used that attribute to discern the effect of arginine substitution

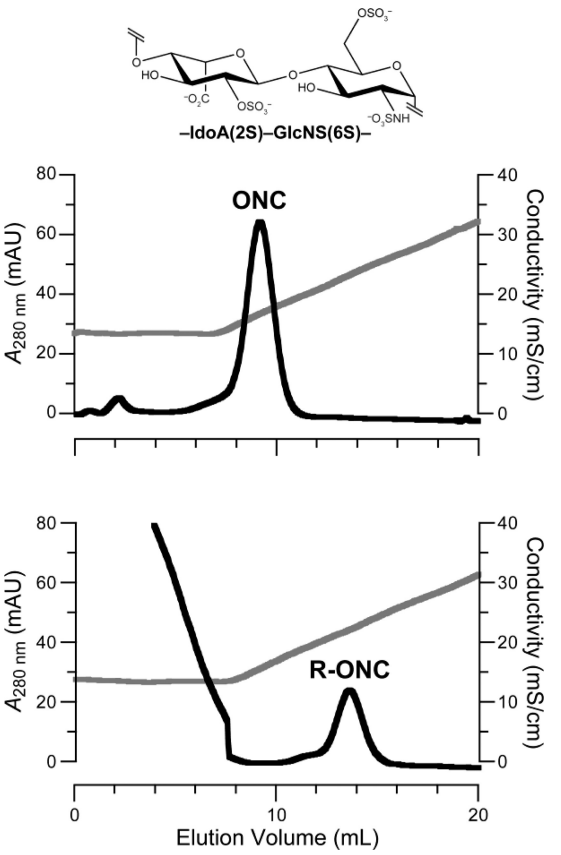

Figure 3. Elution profiles of ONC and R-ONC from immobilized heparin. Ribonucleases were detected by their absorbance at 280 nm (black line) during elution with a linear gradient of NaCl in PBS. Conductivity (gray line): PBS, 14 mS/cm; ONC elution, 16 mS/cm; R-ONC elution, 23 mS/cm. The structure depicts a common disaccharide unit in heparin.

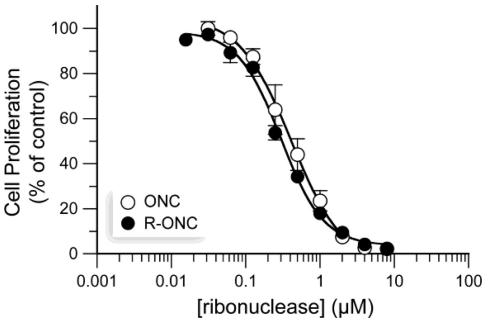

Figure 4. Effect of arginine residues on the cytotoxicity of Onconase. Cellular proliferation was assessed by measuring the incorporation of [*methyl*-^{3}H]thymidine into DNA of K-562 cells in the presence of ribonucleases. IC_{50} values: ONC, 0.4 ± 0.1 μM; R-ONC, 0.29 ± 0.01 μM.

on conformational stability. We found that ONC has ΔG^{H_2O} = 55 ± 7 kJ/mol and $[D]_{50\%}$ = 4.44 ± 0.03 M, whereas R-ONC has ΔG^{H_2O} = 46 ± 9 kJ/mol and $[D]_{50\%}$ = 4.06 ± 0.06 M (Figure 5). These data indicate that arginine substitution destabilizes ONC.

Protease Susceptibility. Finally, we determined whether the decrease in conformational stability of R-ONC might affect its integrity *in cellulo*. To discern the susceptibility of ONC and R-ONC to degradation by proteases, we incubated the protein

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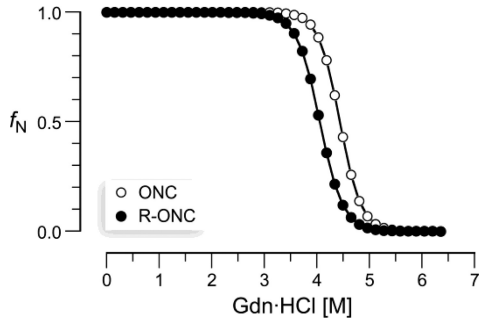

Figure 5. Effect of arginine residues on the conformational stability of Onconase. The guanidine-HCl transition curves of ONC and R-ONC were determined by using fluorescence spectroscopy. f_N refers to the fraction of protein in the native state. $[D]_{50\%}$ values: ONC, 4.44 ± 0.03 M; R-ONC, 4.06 ± 0.06 M.

with pepsin. ONC was resistant to degradation by pepsin over the entire time course of the incubation, remaining 90% intact after 22 h (Figure 6). By contrast, R-ONC was highly susceptible to degradation—only ~25% remained intact after 22 h.

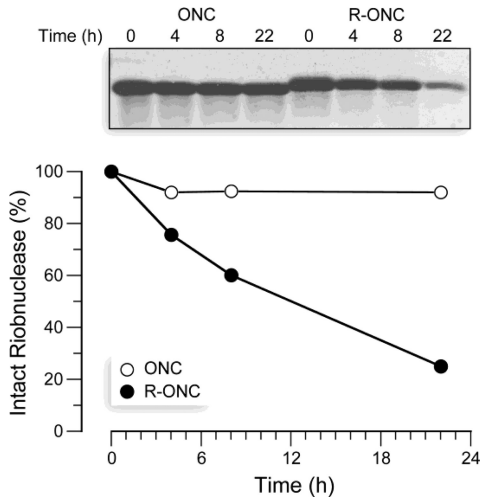

Figure 6. Effect of arginine residues on the protease susceptibility of Onconase. ONC and R-ONC were incubated with pepsin in 50 mM glycine-HCl buffer at pH 2.4 and 37 °C. Aliquots were removed at 0, 4, 8, and 22 h. Samples were analyzed with SDS–PAGE, and stained bands were analyzed by densitometry.

■ DISCUSSION

ONC is a member of the pancreatic-type ribonuclease family with innate antitumoral activity.[3−7] Because it lacks a cell-surface receptor and elicits a distinctive phenotype—cell death—ONC is an ideal model for assessing biochemical attributes that promote the cellular uptake of proteins. Here, we show that arginine substitution leads to an increase in the cellular uptake of ONC.

The cell surface is highly anionic, containing phospholipids, glycosaminoglycans, and heparan sulfate. Though similar in net charge, cell-penetrating peptides with many arginine residues display more efficient cellular uptake than do their lysine counterparts. The guanidinium group of arginine is able to form additional hydrogen bonds with the prevalent carboxyl, phosphoryl, and sulfuryl groups on the cellular surface,[42] which could be responsible for the greater affinity of R-ONC than ONC for heparin (Figure 3). Moreover, arginine is accommodated near the middle of a lipid bilayer with less energetic cost than lysine,[43] and arginine unlike lysine can be charged or uncharged there due to the induction of large water defects in a host membrane.[44]

R-ONC contains 13 arginine and 2 lysine residues. The increase in cellular uptake of R-ONC, though significant, is ~10-fold lower than the levels of cellular uptake of human pancreatic ribonuclease (RNase 1), which has 10 arginine and 8 lysine residues, or of RNase A, which has 4 arginine and 10 lysine residues.[2,27] The distribution of cationic residues could explain the discrepancy in cellular internalization. For example, the cationic residues in RNase 1 and RNase A are clustered in the active-site cleft, whereas those in ONC are distributed across the protein surface.

ONC is an exceptionally stable protein with T_m > 90 °C.[33,34,45] Single amino acid substitutions in ONC, such as F28T, can result in a decrease in conformational stability of >20 kJ/mol.[34] The E49R and D53R substitutions in RNase A, which enhance cellular uptake, decrease the value of T_m by 10 °C.[26] Yet, we find that replacing 10% of the residues in ONC diminishes conformational stability by <10 kJ/mol, a quantity that lacks statistical significance. The small observed decrease in conformational stability is surprising in light of the difficulty that we experienced in folding R-ONC *in vitro* (*vide supra*). Moreover, we were unable to determine a T_m value for R-ONC because of its irreversible denaturation and its precipitation when unfolded (data not shown), in contrast to the behavior of wild-type ONC or other variants.[27,33] We note that another member of the pancreatic-type ribonuclease superfamily, eosinophil cationic protein, also contains more arginine than lysine residues and also unfolds irreversibly *in vitro*.[46]

R-ONC showed a small decrease in conformational stability and a large increase in protease susceptibility. Protease resistance is an important determinant of cytotoxicity.[45,47] Several workers have hypothesized that the resistance of ONC to proteolysis is due to its structural rigidity.[48,34] In particular, Di Donato and co-workers found that the M23L substitution, which disrupts the hydrophobic packing of ONC, has effects on protease susceptibility, catalytic activity, and thermodynamic stability comparable to those in R-ONC.[45] The M23L substitution increases the mobility of the 24–31 loop, which increases its vulnerability to proteases but enables Lys31 to extend further into the active site. An increase in the mobility of the 24–31 loop due to the K33R substitution could explain the increase in catalytic activity but is unlikely to have resulted in all of the changes observed herein. The multiple arginine residues in R-ONC could increase the access of a protease to peptide bonds because interactions of the main chain with the side chain of arginine are more likely to be disruptive than are those with the side chain of lysine.[18]

Because the internalization of R-ONC was 3-fold greater than that of ONC (Figure 2), we expected R-ONC to display a comparable increase in cytotoxicity. Surprisingly, R-ONC remained as cytotoxic as ONC (Figure 4). The observed affinity of R-ONC for heparin (Figure 3) could be indicative of a slight alteration in intracellular routing. Also, replacing lysine residues could render R-ONC less vulnerable to ubiquitination and subsequent proteasomal degradation. More concretely, however, our proteolytic degradation assays showed that the amount of intact R-ONC at 4 h is less than that of intact ONC at 22 h (Figure 5). Apparently, R-ONC maintains its

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cytotoxicity through its greater cellular internalization and higher catalytic activity.

Understanding the tumoral targeting and dose-limiting renal toxicity of ONC is important for the development of more effective chemotherapeutic agents based on pancreatic-type ribonucleases. The renal toxicity of ONC can be attributed to its accumulation in the kidneys, combined with its low protease susceptibility. R-ONC maintains high cytotoxicity but could exhibit less renal toxicity because of its increased protease susceptibility. Relevant studies on the consequences of this interdependence are ongoing in our laboratory.

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ABBREVIATIONS

6-FAM, 6-carboxyfluorescein; ONC, Onconase (a registered trademark of Tamir Biotechnology, Inc.: otherwise, ranpirnase); Pyr, pyroglutamate; GAG, glycosaminoglycan; RNase A, bovine pancreatic ribonuclease; TAMRA, 6-TAMRA, 6-carboxytetramethylrhodamine; *Z*, net molecular charge: Arg + Lys − Asp − Glu − Pyr.

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