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Potentiation of ribonuclease cytotoxicity by a poly(amidoamine) dendrimer

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ABSTRACT

Variants of bovine pancreatic ribonuclease (RNase A) engineered to evade the endogenous ribonuclease inhibitor protein (RI) are toxic to human cancer cells. Increasing the basicity of these variants facilitates their entry into the cytosol and thus increases their cytotoxicity. The installation of additional positive charge also has the deleterious consequence of decreasing ribonucleolytic activity or conformational stability. Here, we report that the same benefit can be availed by co-treating cells with a cationic dendrimer. We find that adding the generation 2 poly(amidoamine) dendrimer in *trans* increases the cytotoxicity of RI-evasive RNase A variants without decreasing their activity or stability. The increased cytotoxicity is not due to increased RI-evasion or cellular internalization, but likely results from improved translocation into the cytosol after endocytosis. These data indicate that co-treatment with highly cationic molecules could enhance the efficacy of ribonucleases as chemotherapeutic agents.

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Pancreatic-type ribonucleases can be toxic to cancerous cells.[1–5] This cytotoxicity depends on the efficiency of their internalization into cells and subsequent translocation into the cytosol, their conformational stability, their ability to evade the endogenous ribonuclease inhibitor protein (RI[6]), and their ability to catalyze the degradation of cellular RNA.[7] Although Onconase®,[8] a ribonuclease from the Northern Leopard frog that naturally evades RI, is in a Phase IIIb clinical trial as a treatment for malignant mesothelioma, its therapeutic potential is diminished by dose-limiting renal toxicity.[9] Mammalian pancreatic ribonucleases accumulate to a much lesser extent in the kidneys, but are strongly inhibited by RI.[9–11] Variants of both bovine (RNase A[12]) and human (RNase 1) pancreatic ribonuclease have been engineered to evade RI and are thereby endowed with cytotoxic activity.[10,11,13,14] An RNase 1 variant is in a Phase I clinical trial for patients having advanced, refractory, solid tumors.

Although ribonucleases show promise as cancer chemotherapeutic agents, their potency is limited by the bottleneck of cellular internalization and translocation into the cytosol. For example, RNase A has cytotoxicity at much lower (e.g., picomolar) concentrations when injected directly into cells.[15,16] RNase A and its homologs are cationic proteins (Fig. 1). Internalization has been shown to correlate with ribonuclease cationicity,[17] and efforts have been made to add positive charge and thereby enhance cytotoxicity. 'Arginine-grafting' and appending a nonaarginine tag do indeed increase the cytotoxicity of RI-evasive RNase A variants.[18,19] These alterations, however, tend to diminish the conformational stability of the ribonuclease, abrogating some of the benefit from increased internalization. RNase A has also been modified by condensing the side chains of aspartate and glutamate residues with ethylenediamine or cationic polymers of poly(ethyleneimine).[21,22] Although these covalent modifications increase cytotoxicity, they diminish catalytic activity. We sought a means to increase ribonuclease-mediated cytotoxicity without compromising other desirable attributes.

To avoid the deleterious consequences of adding cationic groups to a ribonuclease, we hypothesized that ribonuclease cytotoxicity could be enhanced by adding a highly cationic molecule as a co-treatment, that is, in *trans*. This strategy was inspired by the recent observation that co-treatment with a cationic peptide enhances the cellular uptake of proteins.[23] As our co-treatment, we chose to

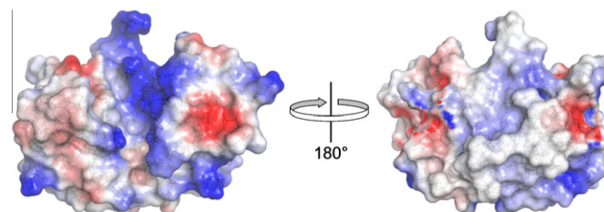

Figure 1. Electrostatic potential map of the RNase A surface with positively charged surface in blue, negatively charged surface in red, and neutral surface in white. Images were created with PDB entry 7rsa[20] and the program PYMOL (DeLano Scientific, San Francisco, CA, USA).

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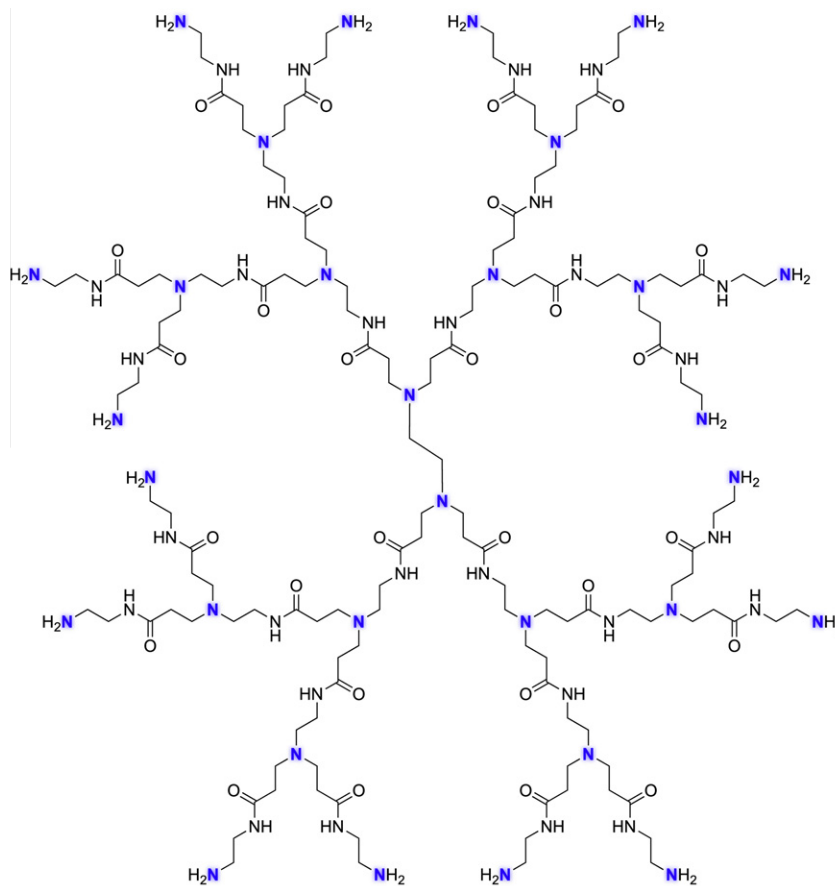


Figure 2. Structure of the generation 2 poly(amidoamine) (PAMAM) dendrimer. The 30 amino nitrogens are in blue.

use the generation 2 poly(amidoamine) (Fig. 2) (PAMAM) dendrimer, which has a high density of cationic charge. PAMAM dendrimers are monodisperse branched polymers that are available from numerous commercial vendors.[24,25] PAMAM dendrimers have been employed as drug and gene delivery vehicles, as they have the ability to transport encapsulated small molecules and enwrapped DNA into cells.[25–28] We chose the relatively small generation 2 dendrimer due to its low inherent cytotoxicity compared to higher generation dendrimers and other highly cationic macromolecules, such as polyethylenimine.[29–31] This dendrimer has 16 primary amino groups and 14 tertiary amino groups. Its net molecular charge is approximately $Z = +16$,[32] and could reach $Z = +30$ in an acidic environment. The dendrimer is known to enter cells through endocytosis and then appears to disrupt endosomes through the 'proton sponge' effect, translocating into the cytosol.[33] We reasoned that dendrimers could elicit an adventitious increase in the translocation of ribonucleases into the cytosol and, thus, in their cytotoxic activity.

Ribonucleases tend to lose conformational stability[18,19] or catalytic activity[21,22] when modified covalently to be more cationic. Accordingly, we first sought to discern whether the dendrimer elicited any effects on these important attributes in *trans*. We found that the dendrimer at concentrations of 1 or 10 μM has no discernable effect on the thermostability of RNase A (Table 1). Similarly, these concentrations had an insignificant effect on ribonucleolytic activity. Accordingly, we conclude that the dendrimer at a concentration of ⩽10 μM has no deleterious consequences.

Large cationic molecules antagonize the RI–RNase A interaction.[34] Hence, we next sought to determine whether the dendrimer is an antagonist of this interaction. Serial dilutions of a solution of dendrimer were used to compete with a 2′,7′-diethylfluorescein-labeled variant of RNase A for binding to RI. Using this assay,[35] we determined that the RI·dendrimer complex in PBS has $K_d = (3.1 \pm 0.5)$ μM, which is significantly greater than RI-evasive variants of RNase A.[10] This value, along with the known concentration of RI in the cytosol (4 μM[36]), indicate that the dendrimer is unlikely to act by antagonizing the RI–RNase A interaction.

Table 1
Effect of dendrimers on RNase A thermostability, and catalytic activity, and cellular internalization

Additive	T_m[a] (°C)	k_{cat}/K_M[b] (10^6 M^{-1} s^{-1})	Internalization (counts)[c]
None	62.7 ± 0.1	33 ± 5	129 ± 9
1 μM dendrimer	62.8 ± 0.1	34 ± 4	125 ± 1
10 μm dendrimer	63.2 ± 0.2	28 ± 2	113 ± 7

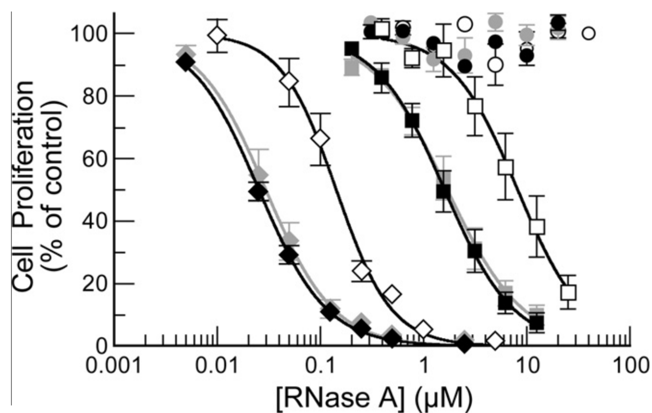
[a] Values of T_m (±SE) were determined in PBS by UV spectroscopy.
[b] Values of k_{cat}/K_M (±SE) were determined for catalysis of 6-FAM–dArUdAdA–6-TAMRA cleavage.
[c] Values of internalization represent the mean (±SE) from three separate experiments performed in triplicate in which K-562 cells were incubated with an esterase-activatable fluorogenic label for 3 h, and internalization was assessed with flow cytometry . The effect of dendrimer on thermostability, catalytic activity, or internalization was not significant (p >0.05).

Having established that the dendrimer is inert in vitro, we sought to test our hypothesis in cellulo. Specifically, we determined the effect of the dendrimer on the cytotoxicity of wild-type RNase A and two RI-evasive variants. We did these experiments with K-562 cells, which are nonadherent cells from a human myelogenous leukemia line. We choose dendrimer concentrations of 1 and 10 μM because these concentrations did not compromise important attributes of the ribonucleases (Table 1) and allowed for the unrestricted proliferation of K-562 cells (97 ± 3% and 96 ± 3%, respectively).

We found that wild-type RNase A showed no measurable cytotoxicity with or without dendrimer (Fig. 3), as expected from its high affinity for RI. In contrast, both 1 and 10 μM dendrimer potentiated the cytotoxicity of two RI-evasive ribonuclease variants, with 10 μM being slightly more effective. At 10 μM dendrimer, G88R RNase A, which is moderately evasive to RI, exhibited a decrease in IC_{50} value of fivefold. D38R/R39D/N67R/G88R (DRNG) RNase A, which is highly evasive to RI and hence more cytotoxic than the G88R variant,[10] likewise had its cytotoxicity potentiated by fivefold. Because the dendrimer evoked the same relative increase in cytotoxicity for both variants, we concluded that the dendrimer acts by enhancing either cellular internalization or translocation.

Ribonucleases enter the cytosol by first being internalized into endosomes and then translocating into the cytosol.[7] To discern whether the dendrimer affected internalization, we attached a fluorogenic label to RNase A, as described previously.[37] This label is not fluorescent until entering endosomes, which contain esterases that unmask its fluorescence. K-562 cells were incubated with or without dendrimer (1 or 10 μM), and ribonuclease internalization was measured by flow cytometry. Dendrimer did not increase internalization of the labeled ribonuclease into the cell (Table 1). Apparently, the dendrimer acts downstream of internalization, probably by increasing endosomal escape.

We conclude that a cationic dendrimer can potentiate the cytotoxicity of RI-evasive variants of RNase A in *trans*. This ability obviates the deleterious consequences that typically accompany chemical or mutagenic modification. Although it is unlikely that the particular strategy described here could be used in a clinical setting, our findings do encourage further exploration, both on the use of cationic dendrimers as co-treatments with chemotherapeutic agents and on the mechanism of their action in this context.



	RNase A	[dendrimer] (μM)	IC_{50} (μM)
○	Wild-type	0	>25
● (gray)	Wild-type	1	>25
●	Wild-type	10	>25
□	G88R	0	8 ± 1
■ (gray)	G88R	1	1.7 ± 0.1
■	G88R	10	1.6 ± 0.1
◇	DRNG	0	0.14 ± 0.01
◆ (gray)	DRNG	1	0.030 ± 0.002
◆	DRNG	10	0.025 ± 0.001

Figure 3. Effect of generation 2 PAMAM dendrimer on the inhibition of cancer cell proliferation by ribonucleases. The proliferation of K-562 cells was measured by the incorporation of [*methyl*-^{3}H]thymidine. Data points represent the mean (±SE) of three separate experiments performed in triplicate.

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Supplementary data

Supplementary data (experimental procedures and RI–dendrimer binding data) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.028.

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