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Site-specific folate conjugation to a cytotoxic protein

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ABSTRACT

Conjugation to folic acid is known to enhance the uptake of molecules by human cells that over-produce folate receptors. Variants of bovine pancreatic ribonuclease (RNase A) that have attenuated affinity for the endogenous ribonuclease inhibitor protein (RI) are toxic to mammalian cells. Here, the random acylation of amino groups in wild-type RNase A with folic acid is shown to decrease its catalytic activity dramatically, presumably because of the alteration to a key active-site residue, Lys41. To effect site-specific coupling, N^{δ}-bromoacetyl-N^{α}-pteroyl-L-ornithine, which is a folate analogue with an electrophilic bromoacetamido group, was synthesized and used to *S*-alkylate Cys88 of the G88C variant of RNase A. The pendant folate moiety does not decrease enzymatic activity, enables RI-evasion, and endows toxicity for cancer cells that over-produce the folate receptor. These data reveal a propitious means for targeting proteins and other molecules to cancer cells.

Secretory ribonucleases such as bovine pancreatic ribonuclease (RNase A[1]; EC 3.1.27.5) can enter cells and evince a cytotoxic enzymatic activity.[2–4] This attribute is rare amongst mammalian proteins. In response, cells have evolved a femtomolar proteinaceous antagonist, the ribonuclease inhibitor (RI), to mitigate damage from secretory ribonucleases that enter the cytosol.[5,6] Although wild-type RNase A is not cytotoxic, variants that evade RI display notable cytotoxic activity.[7,8]

The toxicity of ribonucleases is limited by the amount of enzyme that reaches the cytosol.[9,10] For example, microinjection experiments show that RNase A is $\sim 10^6$-fold more toxic when injected than when added to the extracellular medium.[11] Increasing the positive charge on RNase A by mutagenesis or chemical modification enhances its internalization and cytotoxicity.[12–14] We sought a more precise means to deliver ribonucleolytic activity to the RNA of cells, especially those related to human disease.

Folic acid (vitamin B_9) mediates much of one-carbon metabolism, including the synthesis, repair, and methylation of DNA. Receptors for folic acid are over-produced on the surface of many types of cancer cells, such as those derived from ovarian, endometrial, and brain carcinomas.[15,16] Attachment of folate to small molecules, liposomes, and proteins can enhance their cellular internalization.[17] We reasoned that the conjugation of folate to a ribonuclease could enhance its binding and uptake by cells that over-produce the folate receptor, endowing toxic activity towards those cells.

Historically, the coupling of folate to proteins has relied on nonspecific acylation reactions that lead to a heterogeneous mixture of conjugates.[18] Such random conjugation can affect adversely the activity of a toxin.[19] Herein, we describe the design, synthesis, and use of a new folate analogue equipped for facile attachment to a sulfhydryl group, as is found in the side chain of a cysteine residue. We find that coupling a single folate moiety can render wild-type RNase A into a cytotoxin.

We designed folate analogue **1** for site-specific conjugation to a free cysteine residue in a protein. Our synthetic route to analogue **1** utilized the clever method of Fuchs and co-workers to excise an activated pteroyl group from folic acid.[20] We then availed solid-phase chemistry to ease purification and avoid confounding insolubility while reinstalling the α-carboxyl group along with an electrophilic bromoacetamido group in place of the nonessential[21,17] γ-carboxyl group. We synthesized pteroyl azide in four steps according to the method of Fuchs and co-workers (Fig. 1).[20] Next, we coupled the pteroyl azide to L-ornithine on a resin. Finally, we N-acylated the resin-bound pteroyl-L-ornithine with bromoacetylbromide. Cleavage from the resin provided folate analogue **1**.

We prepared ribonuclease–folate conjugates in two distinct ways: random and site-specific. For random conjugation, folate itself was activated with the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (EDC). Then, a slight

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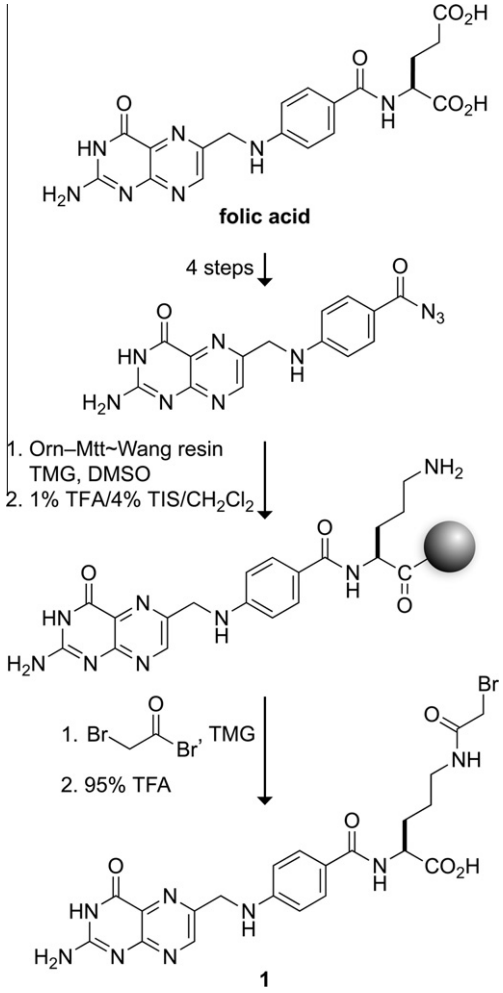

Figure 1. Scheme for the synthesis of N^{δ}-bromoacetyl-N^{α}-pteroyl-L-ornithine (**1**). Pteroyl azide was synthesized from folic acid in four steps as described previously.[20]

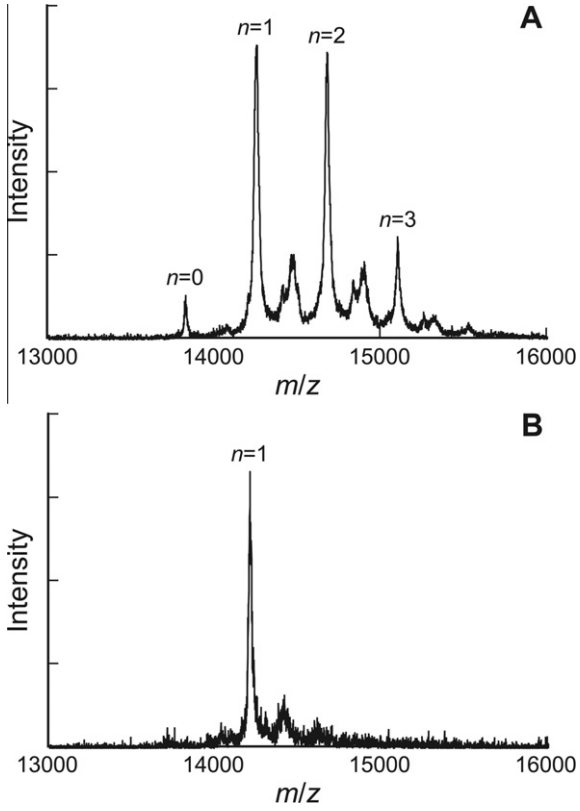

Figure 2. Mass spectra of random and site-specific ribonuclease–folate conjugates. (A) Mass spectrum of K41R/G88R–folate, which was prepared by the random conjugation of folic acid. (n = 1, m/z 14,250; expected: 14,232). (B) Mass spectrum of G88C–folate, which was prepared by site-specific conjugation of folate analogue **1** (m/z 14,205; expected: 14,195). Major peaks are labeled with the number of folate moieties, n. Minor peaks are photochemically generated adducts of the matrix (3,5-dimethoxy-4-hydroxycinnamic acid) with the proteins.

excess of the activated folate was added to RNase A variants to acylate primary amino groups randomly with folate. In addition to the randomness in their site(s) of conjugation, conjugates produced by this method were found to be heterogeneous in the number of folates conjugated per protein (Fig. 2). The catalytic activity of secretory ribonucleases is necessary for their cytotoxicity.[23] RNase A has amino groups in the side chain of 10 lysine residues and at its N-terminus. Lys7, Lys41, and Lys66 are active-site residues that are important for catalysis.[24,25] The side-chain amino group of Lys41 has a low pK_a value[26] and thus suffers especially rapid acylation. Its modification can reduce catalytic proficiency by 10^5-fold.[24] Indeed, we found that RNase A subjected to random acylation by folic acid retains only 0.4% of its catalytic activity (Table 1). Hence, we also attached folate randomly to K41R RNase A, wherein the guanidino group in the side chain of Arg41 retains a positive charge but acts, in effect, as a protecting group that precludes acylation by an activated folate. The K41R variant has 56-fold less catalytic activity than does wild-type RNase A, but retains 54% of its catalytic activity upon conjugation (Table 1). Accordingly, the catalytic activity of K41R–folate is 2-fold greater than that of wild-type-folate. Finally, we attached folate randomly to K41R/G88R RNase A. Gly88 is at the RI–RNase A interface (Fig. 3). The G88R substitution diminishes the affinity for RI by 10^4-fold.[27] As with the K41R variant, K41R/G88R RNase A suffers only a modest decrease in enzymatic activity upon folate conjugation (Table 1).

Table 1
Enzymatic activity of RNase A variants and folate conjugates

Protein/conjugate	k_{cat}/K_M[a] (10^6 M^{-1} s^{-1})
Wild-type	3.0 ± 0.2
Wild-type-folate	0.013 ± 0.006
K41R	0.054 ± 0.003
K41R–folate	0.029 ± 0.006
K41R/G88R	0.055 ± 0.003
K41R/G88R–folate	0.044 ± 0.005
G88R	1.9 ± 0.2
G88C–folate	4.3 ± 0.7
A19C–folate	3.9 ± 0.4

[a] Values of k_{cat}/K_M (± SE) were determined for catalysis of 6-FAM–dArUdAdA–6-TAMRA cleavage in 0.05 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M) at 25 °C.[22]

In contrast to lysine, cysteine is rare in proteins.[28] Moreover, cysteine residues are often engaged in disulfide bonds, which protect them from alkylation. For example, RNase A has eight cysteine residues that form four disulfide bonds. Accordingly, a cysteine residue installed by site-directed mutagenesis in RNase A would display the only enzymic sulfhydryl group.

For site-specific conjugation, we used folate analogue **1** to *S*-alkylate a cysteine installed as residue 19 or 88 in RNase A. The folate installed at residue 88 served a dual purpose—target the conjugate to a cell-surface folate receptor and endow evasion of cytosolic RI. Unlike residue 88, residue 19 is distal from the RI–RNase A interface (Fig. 3), and installing folate there serves as a

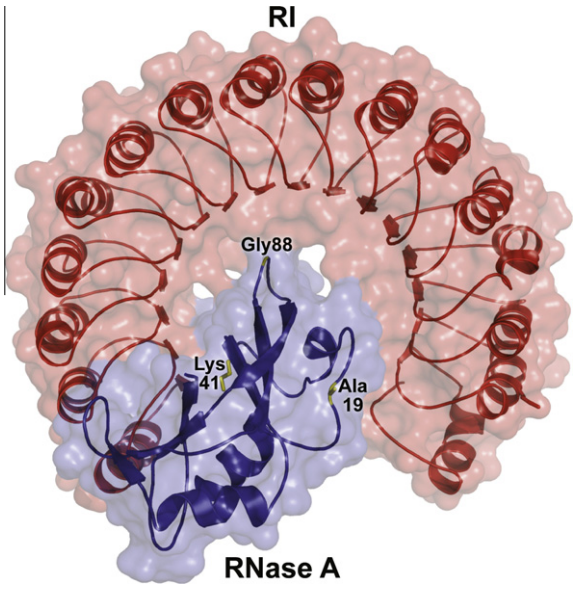

Figure 3. Three-dimensional structure of the complex between RNase A (blue) and RI (red). The location of the three residues substituted herein are shown explicitly. Images were made with PyMol (Delano Scientific) and Protein Data Bank entry 1dfj.[29]

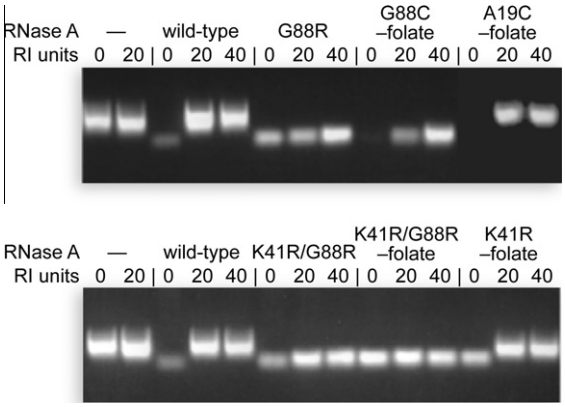

Figure 4. Agarose gel-based assay of the inhibition of the enzymatic activity of RNase A variants and folate conjugates by RI. Inhibition was assessed by visualizing the ribonuclease-catalyzed degradation of 16S and 23S rRNA in the absence or presence of excess RI.[30,27]

control. In contrast to random folate conjugation, site-specific conjugation to RNase A variants with a free cysteine allows conjugates to retain high enzymatic activity (Table 1).

We used an agarose gel-based assay to discern whether a ribonuclease variant or folate conjugate is able to evade RI.[30,27] We found that only those ribonucleases modified by mutagenesis or chemical modification at residue 88 display resistance to inhibition by RI (Fig. 4). These data are expected from the structure of the RI–RNase A complex (Fig. 3).

JAR cells, which are from a placental choriocarcinoma line, overproduce a cell-surface folate receptor when maintained in folate-depleted medium.[16] In preliminary biological experiments, we measured the ability of exogenous folate conjugates to inhibit DNA synthesis within JAR cells. This assay reports, in effect, on the ability of the conjugates to enter the cytosol and catalyze the degradation of cellular RNA, which leads to apoptosis.[31,32] We found that only K41R/G88R–folate (which is a random conjugate) and G88C–folate (which is a site-specific conjugate) inhibit DNA synthesis, with IC_{50} = 11 and 9 μM, respectively. No other conjugate exhibited a detectable effect on DNA synthesis at a concentration of 25 μM. These data are in gratifying agreement with the results shown in Figure 4—only those folate conjugates that have weakened affinity for RI diminish cell proliferation. Apparently, enhanced cellular internalization mediated by a pendant folate moiety must be accompanied by RI-evasion to enable a ribonuclease to be cytotoxic. The ramifications of this duality are the basis for ongoing work in our laboratory.

In summary, we put forth a new folate analogue, **1**, that can be used to install a folate moiety at a designated site in a protein. Unlike random folate conjugation, site-specific folate conjugation need not compromise protein function. Importantly, site-specific conjugation allows for the preparation of a homogeneous toxin, which facilitates molecular characterization and optimization. We anticipate that folate analogue **1** could be used to create useful site-specific folate conjugates of a variety of cytotoxins.

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Supplementary data

Supplementary data (experimental procedures and molecular characterization) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.081.

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