Esterification Delivers a Functional Enzyme into a Human Cell

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⑤ *Supporting Information*

ABSTRACT: A major hurdle in chemical biology is the delivery of native proteins into the cytosol of mammalian cells. Herein, we report that esterification of the carboxyl groups of an enzyme with a diazo compound enables not only its passage into the cytosol but also the retention of its catalytic activity there. This scenario is demonstrated with human ribonuclease 1, which manifests ribonucleolytic activity that can be cytotoxic. After internalization, the nascent esters are hydrolyzed in situ by endogenous esterases, making the process traceless. This strategy provides unprecedented opportunities for the delivery of functional enzymes into human cells.
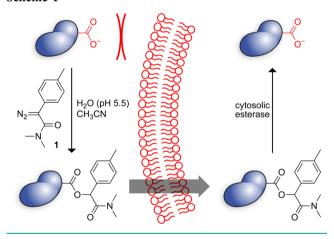

diazo compound

cytosolic esterases

NOT cell-permeable   **cell-permeable**

Biologics are driving the growth of the modern pharmacopeia.[1] Still, nearly all of these biologics are antibodies and hormones that act on the cell surface.[2,3] Virtually none are proteins that intervene beneficially in the cytosol or nucleus, despite incalculable opportunities there.[4]

The translocation of a putative therapeutic agent across the plasma membrane is made difficult by the high anionicity of the glycocalyx and hydrophobicity of the lipid bilayer.[5] Any strategy for protein delivery must overcome these two obstacles. Medicinal chemists have done so by the bioreversible masking of anionic functional groups. The ensuing "prodrugs",[6−8] which are usually esters of carboxylic acids, constitute 20% of small-molecule drugs.[9] Recently, we demonstrated that the esterification of the carboxyl groups of the green fluorescent protein (GFP) with 2-diazo-2-(*p*-methylphenyl)-*N,N*-dimethylacetamide enables its passage across the plasma membrane, akin to a small-molecule prodrug (Scheme 1).[10] Importantly, the nascent esters are substrates for endogenous intracellular esterases.[11,12] Thus, esterification can be a "traceless" delivery method that leaves no residual atoms attached to the cargo.

Upon cytosolic entry, esterified GFP retains its fluorescence,[10] consistent with the retention of its three-dimensional structure.[13] The catalytic activity of an enzyme is, however, the most sensitive indicator of proper conformation.[14] We sought to investigate the utility of esterification as a means to promote the delivery of a functional enzyme.

As a model enzyme, we chose human ribonuclease 1 (RNase 1; EC 3.1.27.5; UniProtKB P07998).[15] RNase 1 is an efficient catalyst of RNA cleavage, and its ribonucleolytic activity can be cytotoxic.[16−18] To be cytotoxic, RNase 1 must not only enter the cytosol but also evade the ribonuclease inhibitor protein (RI) that resides there.[19,20] Wild-type RNase 1 forms a complex with RI that has a K_d value of (at most[21])

Scheme 1

N₂

H₂O (pH 5.5)
CH₃CN

1

cytosolic esterase

0.29 fM[22] and is not appreciably cytotoxic because of its sequestration by RI.[23]

RI is a highly anionic protein, and its evasion is best achieved by installing anionic groups into RNase 1. Indeed, the most evasive known variant is R39D/N67D/N88A/G89D/R91D RNase 1, which has a 10^{10}-fold lower affinity for RI than does the wild-type enzyme.[22] The DDADD variant is, however, not a potent cytotoxin because its anionicity deters cellular uptake.[24] Hence, the DDADD variant along with wild-type RNase 1 were ideal enzymes for the assessment of our approach, using cytotoxicity as a readout.

Table 1. Attributes of Untreated and Esterified RNase 1 Variants

						IC$_{50}$^{e} (μM)	
RNase 1	Z^{a}	carboxyl groups	**1**^{b} (equiv)	esters^c	k_{cat}/K_M^{d} (10^7 M^{-1} s^{-1})	HeLa cells	H460 cells
wild-type	+5	13	0		2.5 ± 0.1	>100	>100
wild-type	+5	13	100	4	1.1 ± 0.5	10 ± 1	7 ± 1
wild-type	+5	13	200	6	1.6 ± 0.1	ND^f	5.1 ± 0.5
DDADD	0	17	0		0.022 ± 0.002	>100	>100
DDADD	0	17	100	7	0.023 ± 0.003	6 ± 1	8.4 ± 0.5
DDADD	0	17	200	11	0.025 ± 0.004	ND	1.0 ± 0.2
H12A/K41A/H119A	+4	13	0		<1 × 10^{-5}	>100	>100
H12A/K41A/H119A	+4	13	100	4	<1 × 10^{-5}	>100	>100
FLAG-labeled	+2	18	0		0.72 ± 0.07	ND	ND
FLAG-labeled	+2	18	100	5	0.35 ± 0.04	ND	ND

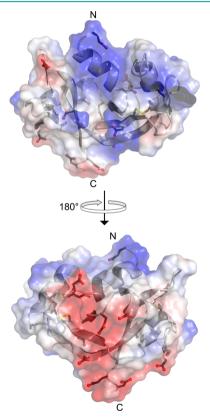^{a}Reference 28. ^{b}Reaction conditions: 200 μL of enzyme (0.14 μmol) in 10 mM MES−HCl buffer (pH 5.5) plus 200 μL of diazo compound **1** (14 or 28 μmol) in acetonitrile; 37 °C for 4 h. ^{c}Values are the most prevalent species apparent in the mass spectra of Figures S3−S6. ^{d}Values are the mean ± SD for the cleavage of 6-FAM−dArU(dA)$_2$−6-TAMRA in 100 mM Tris−HCl buffer (pH 7.5), containing NaCl (100 mM). Individual values are shown in Figure S7. ^{e}Values are the mean ± SD from data in Figures 2 and 3. ^{f}ND, not determined.

The mechanism of esterification with a diazo compound relies on a carboxyl group being in a protonated state.[25,26] To encourage protonation, we performed esterification reactions at pH 5.5 in aqueous acetonitrile (Scheme 1). These conditions were well-tolerated by RNase 1 and its variants and enabled esterification of one-third to one-half of enzymic carboxyl groups (Table 1). Esterification did reduce ribonucleolytic activity slightly (Table 1) but to a lesser extent than did the amidation of enzymic carboxyl groups.[27]

As observed previously,[23] wild-type RNase 1 had no detectable effect on the viability of HeLa cells, even at a concentration of 100 μM (Figure 2A). In contrast, esterification of wild-type RNase 1 engendered toxicity toward HeLa cells with an IC$_{50}$ value of 10 ± 1 μM (Figure 2A). These data are consistent with esterified RNase 1 overwhelming cytosolic RI, which is present at ~4 μM.[29] Notably, only ~7% of unmodified RNase A (which is a bovine homologue of RNase 1) translocates to the cytosol from endosomes.[30] Accordingly, the lack of toxicity of unmodified RNase 1 along with the near-equivalence of the IC$_{50}$ value of esterified RNase 1 and the cytosolic concentration of RI suggests that esterification of RNase 1 makes its cytosolic delivery efficient.

Wild-type RNase 1 has a net charge of Z = 5.[28] The unmodified enzyme enters endosomes rapidly.[24] In contrast, DDADD RNase 1 has a net charge of Z = 0 and enters endosomes slowly.[24] As expected, DDADD RNase 1 did not have an impact on the viability of HeLa cells (Figure 2B). In contrast, esterification made the DDADD variant cytotoxic with an IC$_{50}$ value of 6 ± 1 μM (Figure 2B), despite its low k_{cat}/K_M value (Table 1). This cytotoxicity is consistent with the esterified enzyme entering the cytosol and cleaving cellular RNA there.

We were aware that the observed cytotoxicity of the esterified enzymes could be due to a property other than their catalytic activity. For example, some proteins and peptides are cytotoxic because of their ability to disrupt lipid bilayers.[31,32] To test this alternative mechanism, we employed H12A/K41A/H119A RNase 1, which has an eviscerated active site and no detectable ribonucleolytic activity (Table 1). We found that this variant is not cytotoxic, even upon esterification (Figure 2C). Accordingly, we conclude that the cytotoxicity of both esterified wild-type RNase 1 and esterified DDADD RNase 1 relies on the manifestation of their catalytic activity
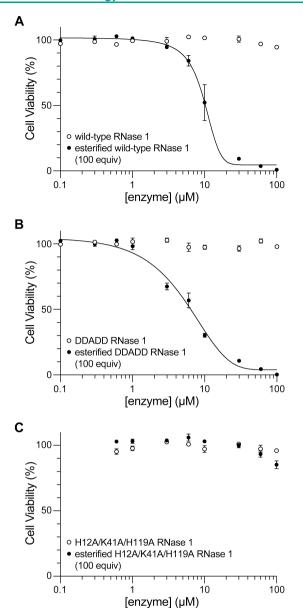

Figure 1. Surface electrostatic potential of human RNase 1 (blue, positive; red, negative). The side chains of the six aspartate, six glutamate, and four cystine residues are shown explicitly. The image was created with the program PyMOL from Schrödinger (New York, NY) and Protein Data Bank entry 1z7x, chain X.[22]

within cells. We note, too, that the toxicity of these esterified enzymes for HeLa cells exceeds that of QBI-139 (IC$_{50}$ = 18 ± 2 μM),[23] which is an RI-evasive variant of RNase 1 that is undergoing clinical trials as a cancer chemotherapeutic agent.[33,34]

HeLa cells, which were derived from a cervical tumor, have numerous abnormalities.[35] Accordingly, we sought to reproduce our results in another cell line. We chose H460 cells, which were derived from a non-small-cell lung tumor. We also used this cell line to assess the effect of esterification level
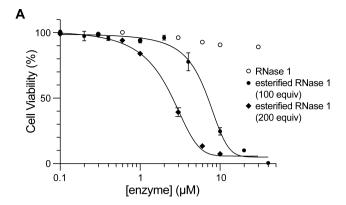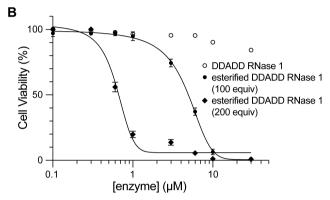
Figure 2. Effect of esterification of human RNase 1 (A) and its variants (B, C) on the viability of HeLa cells. Cell viability was measured with a tetrazolium-dye-based assay for metabolic activity. Values of IC$_{50}$ are listed in Table 1.

Figure 3. Impact of the extent of esterification of human RNase 1 (A) and its DDADD variant (B) on the viability of H460 cells. Cell viability was measured with a tetrazolium-dye-based assay for metabolic activity. Values of IC$_{50}$ are listed in Table 1.

on cytotoxicity. Wild-type RNase 1 and its DDADD variant were treated with either 100 or 200 equiv of diazo compound **1**. We found that increasing the esters in wild-type RNase 1 from ∼4 to ∼6 reduced the IC$_{50}$ value from 7 ± 1 to 5.1 ± 0.5 μM (Figure 3A). Wild-type RNase 1 has 13 carboxyl groups (Figure 1), whereas the DDADD variant has 17 carboxyl groups. The larger number of carboxyl groups amplified the effects. Specifically, we found that increasing the esters in DDADD RNase 1 from ∼7 to ∼11 reduced the IC$_{50}$ value from 8.4 ± 0.5 to 1.0 ± 0.2 μM (Figure 3B).

Finally, we investigated the reversibility of enzymic esterification in living cells. To do so, we appended an eight-residue FLAG tag to the N terminus of wild-type RNase 1 and esterified the resulting FLAG−RNase 1 by using diazo compound **1**. We treated HeLa cells with untreated or esterified FLAG−RNase 1 for 24 h, washed and lysed the cells, and recovered the FLAG−RNase 1 by using anti-FLAG

magnetic beads. Mass spectrometry revealed the removal of labels by intracellular esterases (Figure S6).[36] These data indicate that the esters installed by diazo compound **1** are hydrolyzed by esterases in human cells.

In summary, we have used a diazo compound to esterify enzymic carboxyl groups and have shown that the ensuing enzyme enters the cytosol of human cells and is functional there. Because the catalytic activity of an enzyme is fragile, its maintenance indicates that the delivery process is gentle. Notably, esterification can be performed without the need for mutagenesis,[37] and the modification is traceless, being removed by cellular esterases. This facile, versatile strategy provides new opportunities for delivering native, functional proteins to intracellular targets.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.9b00033.

Experimental procedures, Figure S1 (DNA sequences), Figure S2 (amino acid sequences), and Figures S3−S7 (PDF)

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