Human Ribonuclease with a Pendant Poly(Ethylene Glycol) Inhibits Tumor Growth in Mice

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Abstract

Human pancreatic ribonuclease (RNase 1) is a small secretory protein that catalyzes the cleavage of RNA. This highly cationic enzyme can enter human cells spontaneously but is removed rapidly from circulation by glomerular filtration. Here, this shortcoming is addressed by attaching a poly(ethylene glycol) (PEG) moiety to RNase 1. The pendant has no effect on ribonucleolytic activity but does increase persistence in circulation. The RNase 1–PEG conjugates inhibit the growth of tumors in a xenograft mouse model of human lung cancer. Both retention in circulation and tumor growth inhibition correlate with the size of the pendant PEG. A weekly dose of the 60-kDa conjugate at 1 μmol/kg inhibited nearly all tumor growth without affecting body weight. Its molecular efficacy is ∼5000-fold greater than that of erlotinib, which is a small molecule in clinical use for the treatment of lung cancer. These data demonstrate that the addition of a PEG moiety can enhance the in vivo efficacy of human proteins that act within cells and highlight a simple means of converting an endogenous human enzyme into a cytotoxin with potential clinical utility.

Introduction

An impediment to the development of proteins as chemotherapeutic agents is the dearth of human proteins that can act as cytotoxins. One exception is human pancreatic ribonuclease (RNase 1) [1–3]. Like RNase A (which is its storied bovine homolog [4–6]), RNase 1 catalyzes the cleavage of single-stranded RNA after pyrimidine residues. As a highly cationic protein, RNase 1 can enter the cytosol of human cells spontaneously and there manifest cytotoxic ribonucleolytic activity [7–9]. As a small protein, RNase 1 is cleared rapidly from circulation through glomerular filtration [10,11]. A simple variant of RNase 1 is now in a phase 1 clinical trial as a cancer chemotherapeutic agent [12], and we reasoned that the installation of a poly(ethylene glycol) (PEG) moiety could enhance the utility of RNase 1 by increasing its persistence in circulation [13–15].

RNase 1 has no free cysteine residues. Hence, site-directed mutagenesis can be used to install a unique sulfhydryl group that could be linked to a PEG moiety. Because RNase 1 has 128 residues, the ensuing PEGylated enzyme would be >99% identical in amino acid sequence to an endogenous human protein. Previously, various aspects of this semisynthetic strategy have shown promise with other ribonucleases and nucleases from plants [16–18] and animals [19–22]. Deleterious immunologic responses could, however, compromise the utility of these foreign proteins in the clinic. Accordingly, we sought to validate the strategy with a human enzyme.

Herein, we report on the preclinical attributes of site-specific PEG conjugates to RNase 1. Despite their low cytotoxic activity in vitro, we find that these conjugates are highly efficacious in inhibiting the growth of tumors in xenograft mouse models. This work opens a new frontier in the development of anticancer drugs based on human proteins.

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Materials and Methods

Materials

Escherichia coli BL21(DE3) cells and pET22b(+) plasmid were from Novagen (Madison, WI). Cell lines K-562 (human chronic myelogenous leukemia) and A549 (human alveolar adenocarcinoma) were from the American Type Culture Collection (Manassas, VA). Homozygous male athymic (nu/nu) mice were from Harlan Laboratories (Indianapolis, IN). Cell culture medium and supplements [including Dulbecco’s phosphate-buffered saline (PBS)] were from Invitrogen (Carlsbad, CA).

Erlotinib hydrochloride (Tarceva) was from Genentech (South San Francisco, CA). [Methyl-3H]thymidine (6.7 Ci/mmol) was from PerkinElmer (Boston, MA). RNase 1 substrate 6-FAM-dArUdAdA-H2 was from Integrated DNA Technologies (Coralville, IA). PerkinElmer (Boston, MA). RNase 1 substrate 6-FAM-dArUdAdA-H2 was from Integrated DNA Technologies (Coralville, IA).

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Semisynthesis of RNase 1–PEG Conjugates

RNase 1–PEG conjugates were prepared by S-alkylation of Cys89 of G89C RNase 1 with a maleimido PEG. The pH of the protein solution containing NTB-protected G89C RNase 1 in cation exchange elution buffer was adjusted from 5 to 7.4–8.0 by the addition of 1.0 M Tris-HCl buffer (pH 7.8–8.0) to 20% (vol/vol). The protecting group was removed by adding DTT (5 equiv) and allowing the reaction to proceed for 2 to 5 minutes, resulting in the immediate generation of the yellow TNB anion [29]. The ribonucleases were separated from DTT, TNB, and salt by using a HiTrap desalting column equilibrated with PBS. A 10-fold molar excess of maleimido PEGs 1, 2, or 3 (Figure 1A) was dissolved in an equivalent volume of PBS (relative to the volume of ribonuclease-containing solution) and added to the solution containing the deprotected ribonuclease (final [ribonuclease]: ~200 μM). Alkylation reactions were allowed to proceed at room temperature for ~2 hours or overnight at 4°C. Any remaining maleimido groups were quenched by the addition of DTT to a final concentration of 2 mM. To lessen the concentration of residual salt, reactions were diluted to 50 ml with 50 mM sodium acetate buffer (pH 5.0) and dialyzed overnight against 41 of 50 mM sodium acetate buffer (pH 5.0).
The purification of RNase 1–PEG conjugates relied on the differential affinity for a cation exchange resin. The dialyzed reaction mixture was applied to a column of HiTrap SPHP cation exchange resin equilibrated with the same buffer and eluted from the resin with a linear gradient of NaCl (0–0.65 M) in 50 mM sodium acetate buffer (pH 5.0). Purification of the highly shielded 60-kDa mPEG–G89C RNase 1 required dilution of the dialyzed reaction mixture to reduce the salt concentration. RNase 1–PEG conjugates were purified further by chromatography using HiLoad 26/60 G200 Superdex gel filtration resin equilibrated with 50 mM sodium acetate buffer (pH 5.0) containing NaCl (0.10 M) and NaN₃ (0.05% wt/vol).

**Analyses of RNase 1–PEG Conjugates In Vitro**

**Analytical size exclusion chromatography.** The hydrodynamic volume of the RNase 1–PEG conjugates was estimated with size exclusion chromatography. A 1.0 mg/ml solution of a conjugate in gel filtration buffer was applied to a HiLoad 26/60 Superdex G200 gel filtration column and eluted with 50 mM sodium acetate buffer (pH 5.0) containing NaCl (0.10 M) and NaN₃ (0.05% wt/vol) at a flow rate of 4 ml/min. Gel filtration standards were prepared and separated using the same column according to the guidelines of the manufacturer.

**Assays of catalytic activity.** The ribonucleolytic activity of RNase 1–PEG conjugates was determined by assaying their ability to cleave the hypersensitive fluorogenic substrate 6-FAM-dArUdAdA-6-TAMRA [30]. The MES used to prepare the assay buffer was purified by anion exchange chromatography to remove trace amounts of oligomeric vinylsulfonic acid, which is a by-product of commercial buffer synthesis [31]. The ribonuclease and the pendant PEG. Tumor size was measured twice weekly using calipers, and percent tumor growth inhibition (%TGI) was calculated as described previously [11]. Mice were weighed to reveal severe off-target effects.

**Pharmacokinetic Studies**

Ribonucleolytic activity remaining in the serum of CD-1 mice was used to assess the pharmacokinetic profile of PEGylated ribonucleases. The method was similar to one reported previously [35], except for the assay used to quantify ribonucleolytic activity [22].

**Results and Discussion**

A pendant PEG moiety can confer clinical benefit to several small human proteins, including human growth hormone (Somavert), erythropoietin (Miceria), and interferons α2a (Pegasys) and α2b (Pegintron) [15]. All extant PEGylated drugs, however, act on extra- cellular targets. Manifesting the cytotoxic activity of RNase 1 requires its entry into the cytosol. Thus, for our intent, a pendant PEG moiety could be a “double-edged sword” [36]. The increased hydrodynamic volume imposed by the PEG could enhance persistence in circulation but also hinder the traversal of a lipid bilayer, which is necessary for entry into the cytosol. To probe this dichotomy, we elaborated the G89C variant of RNase 1 with pendant PEGs of different size (Figure 1) and assessed the attributes of the ensuing conjugates both in vitro and in vivo.

**Attributes of RNase 1–PEG Conjugates In Vitro**

A pendant PEG increased the viscosity radius of a ribonuclease (Figure 2). As expected, the viscosity radius correlated with the molecular mass of the PEG moiety: 5 kDa < 20 kDa < 60 kDa.

A small or large PEG moiety at residue 89 had a negligible effect on the ability of RNase 1 to catalyze the cleavage of 6-FAM-dArUdAdA-6-TAMRA.
Table 1. Attributes of RNase 1–PEG Conjugates In Vitro.

<table>
<thead>
<tr>
<th>Ribonuclease</th>
<th>$k_{cat}/K_{M}$ ($10^7$ M$^{-1}$ s$^{-1}$)*</th>
<th>$K_d$ (nM)$^\dagger$</th>
<th>IC$_{50}$ (μM)$^\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type RNase 1</td>
<td>2.9 ± 0.1</td>
<td>2.9 × 10$^{-7}$</td>
<td>&gt;25</td>
</tr>
<tr>
<td>5-kDa mPEG–G89C RNase 1</td>
<td>2.6 ± 0.3</td>
<td>&lt;1.4</td>
<td>&gt;50</td>
</tr>
<tr>
<td>20-kDa mPEG–G89C RNase 1</td>
<td>6.8 ± 0.1</td>
<td>&lt;1.4</td>
<td>&gt;50</td>
</tr>
<tr>
<td>60-kDa mPEG–G89C RNase 1</td>
<td>6.1 ± 0.3</td>
<td>&lt;1.4</td>
<td>&gt;50</td>
</tr>
<tr>
<td>ONC</td>
<td>0.0018 ± 0.00005</td>
<td>150 ± 50</td>
<td>0.22 ± 0.01</td>
</tr>
</tbody>
</table>

*Values of $k_{cat}/K_{M}$ (±SE) are for the catalysis of 6-FAM–dArU(dA)$_2$–6-TAMRA cleavage at room temperature in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M). The values for RNase 1 and ONC were reported previously [11].
†Values of $K_d$ (±SE) are for the complex with human RI at room temperature. The values for wild-type RNase 1 [8] and ONC [37] were reported previously.
‡Values of IC$_{50}$ (±SE) are for the incorporation of [methyl-$^3$H]thymidine into the DNA of K-562 cells. Data are from Figure 3.

(1. Apparently, this distal location does not impede the substrate from gaining access to the active site (Figure 1B). Yet, RI retained notable affinity for the RNase 1–PEG conjugates (Table 1). This sensitivity is likely a result of the extremely high affinity of RI for wild-type RNase 1 [8], as well as the orientation of the side chain of residue 89. Although this residue is at the RI–RNase 1 interface [23], its side chain is oriented nearly perpendicular to the major plane of the RI molecule (Figure 1B).

The RNase 1–PEG conjugates had no discernable effect on the proliferation of K-562 cells in vitro (Table 1 and Figure 3). Likewise, the RNase 1–PEG conjugates had no effect on the proliferation of A549 cells in vitro (data not shown). These data are consistent with a detrimental effect of PEG on membrane translocation, along with the residual affinity for RI, which is known to suppress cytotoxic activity in vitro [7,8].

Efficacy of RNase 1–PEG Conjugates in a Xenograft Model

The three RNase 1–PEG conjugates inhibited the growth of solid human tumors in a mouse xenograft model (Figure 4). Tumor growth inhibition by RNase 1–PEG conjugates was more pronounced at a higher dose and for conjugates with larger PEG moieties (Figure 5). More specifically, values of %TGI correlated with those of viscosity radii (cf. Figures 2 and 5). The enhanced efficacy of the larger conjugates is attributable to their longer persistence in circulation, which is known to correlate with PEG molecular mass [38]. Our pharmacokinetic analyses are consistent with this explanation—larger PEG moieties did indeed increase the persistence of a conjugate in serum (Figure 6). The increased persistence is likely due to a decreased rate of glomerular filtration [39,40].

The ability of the larger conjugates to inhibit tumor growth is even greater than suggested by the data depicted in Figure 4. The indicated
dose (7.5 or 75 mg/kg) includes the mass of the polymer. Because the molecular mass of RNase 1 is 14.6 kDa, a weekly 7.5 mg/kg dose of the 5-, 20-, or 60-kDa PEG conjugate corresponds to only 383, 217, or 101 nmol/kg, respectively. Thus, mice at a particular dosing level received nearly four-fold as many molecules of the 5-kDa PEG conjugate than the 60-kDa PEG conjugate.

Considering the dosage in molecular rather than mass terms also highlights an advantage of using an enzymatic catalyst as a drug, a few molecules can have a large effect. A weekly dose of 7.5 mg/kg of the 60-kDa PEG conjugate inhibited the growth of tumors at a level indistinguishable from that of a biweekly dose of 100 mg/kg erlotinib (Figure 4). This tyrosine kinase inhibitor was approved in 2004 for the treatment of non–small cell lung cancer [41]. Because erlotinib hydrochloride has a molecular mass of 429.90 Da, treated mice received 0.47 mmol/kg each week. Hence, we estimate that 60-kDa mPEG hydrochloride has a molecular mass of 429.90 Da, treated mice received the treatment of nonPEG conjugates (Figure 5).

None of the RNase 1–PEG conjugates exhibited systemic toxicity to the animals as evidenced by their maintenance of body weight throughout the duration of the studies (Figure 5, insets). These data indicate that the toxicity of the RNase 1–PEG conjugates toward neoplastic cells is selective in vivo.

Basis for the Therapeutic Index of RNase 1–PEG Conjugates

RNase 1 conjugated to human antibody fragments that are directed against tumor-associated antigens has shown promise in preclinical studies [42–44]. In those conjugates, specificity derives largely from the antibody fragment, which is also likely to enhance pharmacokinetics. What is the basis for the favorable therapeutic index demonstrated by the RNase 1–PEG conjugates (Figure 5)?

To date, two contributing factors have been implicated for the cancer cell selectivity of RNase 1 and its homologs in vitro. First, cancer cells are more anionic than comparable noncancerous cells [45–47]. Accordingly, Coulomb’s law increases preferentially their uptake of highly cationic molecules, such as RNase 1–PEG conjugates [9,48]. Second, cancer cells have an enhanced rate of endocytosis [49] and could take up RNase 1–PEG conjugates more rapidly than do noncancerous cells.

An additional factor is likely to contribute to the favorable therapeutic index of the RNase 1–PEG conjugates in vivo. The enhanced permeability and retention effect is a universal characteristic of solid tumors [50,51]. Some of the unique aspects of tumor physiology that contribute to the enhanced permeability and retention effect are the increased permeability of tumor vasculature as well as the poor lymphatic drainage of tumor tissues. The ensuing diminished plasma clearance of macromolecules could combine with architectural differences between normal and neoplastic tissues to accumulate RNase 1–PEG conjugates near tumors. In addition, the large size of the conjugates could extend their retention in tumor tissue, as in circulation (Figure 6).

Conclusions

The site-specific conjugation of a PEG moiety is a promising strategy for the development of cancer chemotherapeutic agents based on RNase 1. Attaching a PEG moiety to residue 89 of RNase 1 preserves catalytic activity, and the ensuing RNase 1–PEG conjugates exhibit marked activity for inhibiting the growth of tumors in xenograft mouse models without detectable systemic toxicity. The antitumor activity of these conjugates in vivo as well as their persistence in circulation increases with PEG size, consistent with the passive accumulation in solid tumors as a key to efficacy. This work provides validation for PEGylated human pancreatic ribonucleases as cancer chemotherapeutic agents.
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