ARTICLE

Improving the Pharmacodynamics and In Vivo Activity of ENPP1-Fc Through Protein and Glycosylation Engineering

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Enzyme replacement with ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) eliminates mortality in a murine model of the lethal calcification disorder generalized arterial calcification of infancy. We used protein engineering, glycan optimization, and a novel biomanufacturing platform to enhance potency by using a three-prong strategy. First, we added new N-glycans to ENPP1; second, we optimized pH-dependent cellular recycling by protein engineering of the Fc neonatal receptor; finally, we used a two-step process to improve sialylation by first producing ENPP1-Fc in cells stably transfected with human α-2,6-sialyltransferase (ST6) and further enhanced terminal sialylation by supplementing production with 1,3,4-O-Bu₃ManNAc. These steps sequentially increased the half-life of the parent compound in rodents from 37 hours to ~ 67 hours with an added N-glycan, to ~ 96 hours with optimized pH-dependent Fc recycling, to ~ 204 hours when the therapeutic was produced in ST6-overexpressing cells with 1,3,4-O-Bu₃ManNAc supplementation. The alterations were demonstrated to increase drug potency by maintaining efficacious levels of plasma phosphoanhydride pyrophosphate in ENPP1-deficient mice when the optimized biologic was administered at a 10-fold lower mass dose less frequently than the parent compound—once every 10 days vs. 3 times a week. We believe these improvements represent a general strategy to rationally optimize protein therapeutics.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
✔ The accepted standard for frequency of blood based enzyme therapy is once a day. For example, hemophilia is treated with intravenous doses up to four times a day to control bleeding, whereas asfotase alpha is dosed six times a week subcutaneously.

WHAT QUESTION DID THIS STUDY ADDRESS?
✔ We sought to determine the effect of a rational combinatorial strategy of optimization on drug potency, pharmacokinetics (PKs), and pharmacodynamics. We utilized a three-prong approach, in which we rationally searched for beneficial N-linked glycosylation, and combined these with enhancement of pH dependent recycling of the Fc domain and glyco-polishing techniques implemented during bioproduction.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
✔ Our findings demonstrate that the PKs and potency of blood based enzyme therapeutics can be rationally optimized to enable of sub-milligram/kg bimonthly dosing regimens delivered via subcutaneous injections. Such therapies are routinely used in enzyme replacement therapy (ERT) for metabolic disorders.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
✔ By vastly improving PK and potency of ERT with the methods described, the cost of goods, patient compliance, and therapeutic efficacy of ERT can be greatly improved. We posit that if our results translate into humans, the methods described will shift the impetus for drug development in appropriate metabolic disorders away from gene therapy and toward protein replacement therapy.

The ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) enzyme family catalyzes phosphoryl-transfer reactions on extracellular phosphoanhydrides. The products of these reactions, such as ATP, lysophosphatidic acid, and pyrophosphate, are essential extracellular signaling molecules that govern whole organismal fate through the regulation of essential biological functions, such as angiogenesis,¹⁻³ cell motility,¹⁻⁵ tumor metastasis,⁶⁻¹⁰ bone mineralization,¹¹⁻¹⁴ vascular calcification,¹⁵⁻¹⁷ and hemostasis.¹⁸ Nonenzymatic phosphoryl-transfer reactions that produce these critical signaling molecules have some of the slowest reaction rates known; as a result, living systems

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Levels of PPi in healthy individuals are ~2.0–2.2 μM,20 com-

thal disorders of ectopic calcification in infants to skeletal 

extracellular PPi with deficiencies resulting in potentially le-

phosphates. ENPP1 is the only human enzyme that synthesizes 

pyrophosphate (PPi) from extracellular nucleotide triphos-

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Improving the Potency of ENPP1-Fc
Stabach et al.

Design criteria for addition of new N-glycans to ENPP1-Fc

AUC = C_{max} \frac{k_a}{(k_a - k_e)} \left[ \frac{1}{(k_a)} - 1 \right] = \frac{C_{max}}{k_e} \left[ 1 - \frac{k_a}{k_e} \right] \frac{1}{\left[ 1 - \frac{k_a}{k_e} \right]}

Equation 2: Relationship between AUC, $k_e$, and peak plasma concentration ($C_{max}$).

Mice of the same age and weight were used for all PK experiments, and volume of distribution was assumed to be constant between the animals. Blood samples were collected at 4 time points that initially were between 12 and 75 hours postinjection; later, with PK improvements, time points were taken between 21 and 263 hours postinjection. The initial fractional activity from the first bleed was assigned a fractional activity of 0.9. Mice were also immunosuppressed with an i.p. injection of anti-CD4 clone Gk1.5 24 hours before s.c. injection of enzyme to mitigate any negative immune responses mounted against the longer lasting therapeutic. Plasma PPI was measured in murine plasma as previously described. The data were analyzed and visualized with GraphPad Prism 8.

RESULTS

Design criteria for addition of new N-glycans to ENPP1-Fc

Based on evidence that therapeutic proteins benefit from the addition of N-glycans (e.g., insulin and erythropoietin), we applied this strategy to ENPP1-Fc. Although the general principle of adding an N-glycan to a therapeutic
protein is established, there are no established guidelines to add the glycan for maximal benefit. Nevertheless, we used a semirational approach, which first required knowledge of existing glycans. We began by noting that the crystal structure of murine Enpp1 exhibits four glycans, which we reasoned were also present in (highly homologous) human ENPP1. Human ENPP1 has six N-glycan consensus sequences (N-GCS; Figure S2a), which we incorporated into structural models with genetic data from patients with GACI to help predict beneficial glycovariants. Specifically, we created structural models integrating putative glycosylation sites with the locations of ENPP1 loss of function mutations present in patients with GACI (Figure S2b). We then used the protein sequence database to identify N-GCS in ENPP family members (ENPP2-ENPP7) from all mammalian species with available protein sequences, and modeled these sites onto mouse Enpp1 (PDB 4GTW). Using these tools, we established a list of potential N-GCS sites by avoiding sites near inactivating ENPP1 mutations and sites that would disrupt disulfide crosslinking.

Referencing the list, we introduced additional N-GCS (consisting of Asn-X-Ser/Thr where X is any amino acid except proline) into the parent isoform (hENPP1-Fc, construct #770). We specifically targeted disordered regions on the exterior surface of ENPP1-Fc that permitted the introduction of an N-GCS through a single amino acid substitution. If the location of a proposed N-GCS was near inactivating mutations in patients with GACI or interfered with the disulfide crosslinking, the modifications were not pursued. By using these criteria, we selected 31 possible sites to add N-glycans, attempting to individually sample the entire surface area of ENPP1 (Figure 1a and Table S1). Each of these 31 N-GCS were introduced individually, or in combination, into the parent ENPP1-Fc (construct #770) via site directed mutagenesis, resulting in a final pool of 53 ENPP1-Fc glycoforms (Table S1). Protein production and hydrolytic velocity of the pNP-TMP colorimetric substrate (Figure S3, green bars) was measured in each isoform after transient expression in CHO cells. The nine most promising N-GCS isoforms were then established as stably selected CHO K1 cell clones, and the glycoforms were individually expressed and purified to homogeneity.

Figure 1 Pharmacokinetic effects of additional N-Glycans. (a) Domain structure of the parent clone drawn to scale with the position of all mutations indicated by vertical lines. The two tandem somatomedin B domains (green), catalytic domain (blue) and endonuclease domain (purple), of human ENPP1 was fused N-terminally with the signal sequence of human ENPP7 (cyan) and c-terminally with the Fc domain of human IgG1 (red). The Fc domain contains two sets of mutations; M242Y/S254T/T246E (MST) and H433K/N434F (HN), which were introduced to enhance Fc neonatal receptor (FcRn) mediated endosomal recycling. All other lines indicate attempts to introduce new N-glycosylation sequons (N-GCS) into the protein. Sequons that reduced enzymatic activity in vitro are positioned below the domain structure whereas those that maintained activity are positioned above. Only the I256T mutation, which creates a new glycan at position Asn254, enhanced pharmacokinetic (PK) properties of the protein in vivo. (b) PK analysis of the parent clone 770 tested in 5 animals fitted to curve described in Eq. 1. The fractional enzyme activity, corresponding to the fractional activity compared with peak plasma concentration (C_{max}), was sampled in each mouse at 4 separate times over 200 hours (individual data points overlaid in green). (c) PK effects of additional N-GCS engineered into the parent clone (770). Area under the curve (AUC, left y axis) in blue and half-life (right y-axis) overlayed in red. Error bars represent SD of the mean. Blue and red asterisks represent significance of AUC and half-life measurement, respectively, compared with clone 770. *P < 0.05, ****P < 0.0001, analysis of variance (ANOVA) comparison of means. (d) Steady-state Michaelis–Menten kinetic assays performed at two enzyme concentrations comparing 770 (in black) with clones possessing the I256T mutation (clones 17 in yellow and 19 in red). Each point represents the average of six measurements of each enzyme at each concentration. Error bars denote SDs of the mean, mOD, measured as the optical density; TMP-pNP, thymidine 5’-monophosphate p-nitrophenyl.
PK effects of adding N-glycans to ENPP1-Fc
The half-life and AUC of stably expressed ENPP1-Fc glycoforms were tested in C57BL6 mice using a single subcutaneous injection, with doses varying from 5 mg/kg for the early constructs to 0.3 mg/kg for the latter more potent constructs. The parent ENPP1-Fc (clone 770) yielding a half-life of 37 hours and an AUC of 3,382 mOD/min hour (Table 1, Figure 1b); the effect of adding an additional N-GCS was modest with the notable exception of the I256T mutation, designed to add an N-glycan at asparagine 254 in the catalytic domain near the active site. This N-glycan, introduced because it is present in human ENPP3, increased the AUC of ENPP1-Fc by ~8-fold and half-life by a factor of 1.8 (construct 7, Table 1 and Figure 1c).

To determine whether the ~8-fold increase in AUC resulted from an increased half-life or a gain in catalytic activity, we compared the Michaelis–Menten kinetic constants of the parent ENPP1-Fc with two I256T containing constructs (clone 17 and clone 19) at two different concentrations, and found no significant differences in the K_m or K_cat (Figure 1d). We then confirmed that the mutation resulted in the predicted glycan at Asn254 using mass spectrometry, by identifying that the I256T clone exhibited increased sialyl glycopeptide peaks in the digested peptide fragment 241SGTFFWPGDSVEINTFDPDYK262 compared with the parent ENPP1-Fc (Figure S4). As demonstrated in Figure 2b, the increase in AUC in the I256T clone appears to primarily result from an increased C_max due to the presence of an additional glycosylation.

PK effects of optimizing pH-dependent FcRn recycling
Mutations in the Fc domain of therapeutic antibodies are known to enhance the pH dependent interactions of Fc with neonatal receptor (FcRn) and thereby extend circulatory half-life.37 We examined the effect of two sets of such Fc mutations—H433K/N434F (i.e., “HN” mutations) and M242Y/S254T/T246E (“MST” mutations, Figure S5). The MST-induced changes in the pH dependent affinity of the Fc domain for murine, monkey, and human FcRns are well-documented in the literature.37-39 Both Fc variants were improved because it is present in human ENPP3, in the catalytic domain near the active site. This N-glycan, introduced because it is present in human ENPP3, increased the AUC of ENPP1-Fc by ~8-fold and half-life by a factor of 1.8 (construct 7, Table 1 and Figure 1c).

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Table 1 Pharmacokinetic effects of added N-GCS

| Construct | Signal sequence | Catalytic domain | Nuclease domain | Linker 2 | Half-life, hours | AUC, mOD/min hour |
|-----------|----------------|-----------------|----------------|---------|-----------------|------------------|
| 770       |                |                 |                |         |                 |                  |
| 1         | C25N K27T      | K369N/1371T     | E592N          |         | 37              | 3,382            |
| 2         | C25N K27T      | V29N            | E592N          |         | 40              | 1,935            |
| 3         | C25N K27T      | S766N           | E864N L866T    | 36      | 2,561           |
| 4         | C25N K27T      |                 | S766N          | 36      | 4,134           |
| 5         | C25N K27T      |                 | E864N L866T    | 35      | 4,536           |
| 6         | C25N K27T      |                 | E864N L866T    | 36      | 11,997          |
| 7         | I256T          |                 |                | 66      | 26,596          |

The color shades correspond to the domain coloring of the ENPP1 cartoon presented in Figure 1a, i.e., the listed mutations in the table are colored according to their domain location in the cartoon in Figure 1a.

AUC, area under the curve; mOD, measured as the optical density; N-GCS, N-glycan consensus sequences.
PK effects of increased flux-based sialylation from 1,3,4-O-Bu₃ManNAc

Benefits derived from producing ENPP1 in α-2,6-ST overexpressing CHO cells include a gain in human-like α-2,6-linked sialic acids and an overall increase in sialylation due to the combined effect of α-2,3-sialic and α-2,6-sialic acids, which improves serum half-life by concealing circulating proteins from asialoglycoprotein receptors.41,43 To exploit the benefits of increased sialylation, we supplemented the production cells with 1,3,4-O-Bu₃ManNAc, a “high-flux” metabolic precursor that supplies flux into the sialic acid biosynthetic pathway and increases glycoconjugate sialylation.44,45 This metabolite provided added benefit when incorporated into our biomanufacturing platform, as illustrated by clone 9 (Figure 2c). This ENPP1-Fc glycoform exhibited modestly increased biologic effects when produced in standard CHO cells; AUC increased by 2.5-fold when expressed in CHO cells overexpressing α-2,6-ST, but expression of the clone in cell culture media with 1,3,4-O-Bu₃ManNAc increased AUC an additional ~1.7-fold for a cumulative benefit of 4.3-fold in AUC (clones 9, 9(ST), and 9(ST)A, respectively; Table 3 and Figure 2e). Finally, expressing clone 19(ST) in media containing 1,3,4-O-Bu₃ManNAc yielded the best performing ENPP1-Fc glycoform, increasing AUC by ~13-fold above baseline (clones 770 vs. 19(ST)A, Figure 3a,b). Mass spectrometry analysis confirmed that sialic acid content was increased in 19(ST)A (Figure 3c).

Figure 2 Pharmacokinetic (PK) effects of combining N-glycans, Fc mutations, and glyco-polishing. (a) PK effects of Fc mutations—overlays of area under the curve (AUC; left y axis, blue symbols) and half-life (right y-axis, red symbols). Clones containing the FcH433K/N434F (HN) mutation are in green font and clones containing the Fc-M242Y/S254T/T246E (MST) mutation are in ox-blood font.43 Blue and red asterisks represent significance of AUC and half-life measurements, respectively. (b) Activity vs. time plots of clones 14, 7, and 19 used to derive AUC data. Animals were dosed subcutaneously with 1 mg/kg of each construct. Data points represent means and SDs of individual data displayed in a and c. Increased drug absorption from the subcutaneous depot is reflected by the increased maximal enzyme activity (peak plasma concentration (C_max)) in construct 7 and 19 compared with construct 14. Construct 7 and 19 possess the I256T glycosylation, unlike construct 14. (c) PK effects of glyco-polishing represented by overlays of AUC (left y-axis, blue symbols) and half-life (right y-axis, red symbols). PK constants of glycoforms expressed in unmodified Chinese hamster ovary (CHO) cells—clone numbers only—are compared with those expressed in CHO cells stably transfected with α-2,6-sialyltransferase (α-2,6-ST) with or without 1,3,4-O-Bu₃ManNAc supplementation—clone numbers followed by ST or STA, respectively. To avoid clutter, statistical significance is only denoted in cohorts marked by arrows to highlight two examples of progressive improvements in PK via glyco-polishing discussed in text. (d) Anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) of clone 9 grown in CHO K1 cells alone or stably transfected with human α-2,6-ST or a combination of α-2,6-ST and the sialic acid precursor 1,3,4-O-Bu₃ManNAc shows a progressive increase in the percentage of sialic acid content with each treatment. (e) Activity vs. time plots comparing clone 770 with glycopolished forms of clone 9 to demonstrate the progressive effects of glyco-polishing on C_max and AUC. Animals were dosed subcutaneously with 5 mg/kg of each construct. Data points represent the means and SD of individual data displayed in c. *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.0001, ANOVA comparison of means. mOD, measured as the optical density.
Improving the Potency of ENPP1-Fc

In vivo confirmation of the disease-reversing ability of bio molecularly engineered ENPP1-Fc

The results presented above demonstrate that by evaluating a modestly sized panel of glycosylation-engineered and protein-engineered variants of ENPP1-Fc and by further producing these nascent therapeutics using a novel biomanufacturing platform that ensures a high level of "humanized" sialylation, we were able to identify specific candidates with substantially improved PK properties. To close this study, we confirmed that the newly engineered forms of ENPP1-Fc retained disease-reversing activity in vivo by monitoring PPI in plasma, which is a biomarker for clinical efficacy of ENPP1 enzyme replacement therapy. To compare the PD effects of the above alterations, we dosed Enpp1asj/asj mice with a single subcutaneous dose of 770 and 19(ST) and measured plasma PPI and enzyme activity in plasma for 11 days (Figure 3d). Plasma PPI in mice dosed with parent ENPP1-Fc (clone 770) required a weekly dose of 7.5 mg/kg to maintain plasma PPI.

Table 2 Pharmacokinetic effects combining N-GCS and Fc mutations

| Construct | Signal sequence | Catalytic domain | Nuclease domain | Fc domain | Half-life, hours | AUC, mOD/min hour |
|-----------|----------------|-----------------|-----------------|-----------|-----------------|-----------------|
| 8         |                |                 | S766N           | M883Y S885T T887E | 45              | 1,912           |
| 770       |                |                 | S766N           | H1064K N1065F  | 37              | 3,382           |
| 9         | C25N K27T      | S766N           | H1064K N1065F  | 65        | 6,047           |
| 10        | V29N           | S766N           | H1064K N1065F  | 55        | 7,735           |
| 11        | V29N           | H1064K N1065F  | 57              | 14,506    |
| 12        | V29N           | H1064K N1065F  | 63              | 13,812    |
| 13        | V29N           | E592N           | 70              | 14,978    |
| 14        | V29N           | E592N           | 95              | 20,360    |
| 15        | V29N           | E592N           | 99              | 22,690    |
| 7         | V29N           | I256T           | 66              | 26,596    |
| 17        | V29N           | I256T           | 120             | 33,751    |

The color shades correspond to the domain coloring of the ENPP1 cartoon presented in Figure 1a, i.e., the listed mutations in the table are colored according to their domain location in the cartoon in figure 1a.

AUC, area under the curve; mOD, measured as the optical density; N-GCS, N-glycan consensus sequences.

Table 3 Pharmacokinetic effects of increased and α-2,6-sialylation

| Construct | Signal sequence | Catalytic domain | Nuclease domain | Fc domain | Half-life, hours | AUC, mOD/min hour |
|-----------|----------------|-----------------|-----------------|-----------|-----------------|-----------------|
| 770       |                |                 |                 |           | 37              | 3,381           |
| 1         |                |                 |                 |           | 40              | 1,935           |
| 2         |                |                 |                 |           | 35              | 2,561           |
| 2(ST)     |                |                 |                 |           | 36              | 4,426           |
| 9         | V29N           | K369N/I371T     | S766N           | M883Y S885T T887E | 65              | 6,047           |
| 10        | C25N K27T      | S766N           | H1064K N1065F  | 55        | 7,735           |
| 1(ST)     |                | E592N           | 49              | 8,379    |
| 15(ST)    | V29N           | E592N           | 88              | 13,871   |
| 13        | V29N           | E592N           | 70              | 14,978   |
| 9(ST)     | V29N           | E592N           | 86              | 15,099   |
| 18        | V29N           | E592N           | 83              | 19,638   |
| 14        | V29N           | E592N           | 96              | 20,360   |
| 10(ST)    | C25N K27T      | S766N           | M883Y S885T T887E | 70.1     | 18,207           |
| 15        | V29N           | E592N           | 99              | 22,620   |
| 14(ST)    | V29N           | E592N           | 119             | 22,793   |
| 9(ST) A   | V29N           | S766N           | M883Y S885T T887E | 115     | 26,312           |
| 18(ST) A  | V29N           | E592N           | 97.5             | 14,263 |
| 7         | V29N           | I256T           | 67              | 26,598   |
| 17        | V29N           | I256T           | 120             | 33,752   |
| 19(ST)    | V29N           | I256T           | 170             | 35,252   |
| 19(ST) A  | V29N           | I256T           | 205             | 36,595   |
| 19(ST) A  | V29N           | I256T           | 204             | 44,742   |

The color shades correspond to the domain coloring of the ENPP1 cartoon presented in Figure 1a, i.e., the listed mutations in the table are colored according to their domain location in the cartoon in figure 1a.

AUC, area under the curve; mOD, measured as the optical density.
in the normal range (Figure 3e), whereas a single dose of 0.3 mg/kg of 19(ST) elevated plasma PPI at or above the normal range for ~ 250 hours (Figure 3d), representing a PK gain of ~ 37-fold. Figure 3d also demonstrates that plasma PPI was more variable than plasma enzyme concentration, suggesting a physiological tug-of-war between the PPI generating ENPP1-Fc and the PPI degrading enzyme ALPL, which may elevate when plasma PPI levels are too high.

DISCUSSION

In this study, we sequentially applied strategies to optimize the pharmacologic and PD properties of a therapeutic enzyme designed to treat ENPP1 deficiency while maintaining full catalytic activity. Our approach differed from previous biologic optimization studies in two ways. First, whereas the various strategies (e.g., Fc fusion proteins and mutations, building in N-glycosylation sites, and using downstream biomanufacturing strategies to increase sialylation) have been used individually in the past, they have not been combined to aggregate benefit, and second, glycan engineering and glycopolishing have never been used to optimize enzyme therapeutics. Our studies demonstrate the significant benefit resulting from sequentially combining these complementary strategies in enzyme biologics.

In the current work, our first attempts to improve ENPP1-Fc were inspired by a growing number of reports where addition of N-glycans to therapeutic proteins (so-called “hyper-glycosylation”) improved their effectiveness. Erythropoietin (EPO)
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provides a groundbreaking example; specifically, Aranesp (darbepoetin alfa) is a glycoengineered form of human recombinant EPO where two N-glycans were added to EPO’s three endogenous N-glycans, resulting in an approximately threefold enhancement of serum half-life. In examples that have not yet reached the clinic, a non-canonical N-glycan improves the neutralizing action of an HIV-targeting antibody and N-glycans added to insulin (a normally unglycosylated therapeutic protein) improve its function.

Our efforts to similarly glycoengineer ENPP1-Fc by adding one or more N-GCS yielded negligible benefit with one notable exception. Specifically, we identified a single beneficial N-GCS that provided substantially greater improvement than any of other techniques we used singly—the I256T mutation, which introduced a glycosylation in residue 254. This N-GCS was found by referencing other ENPP family members and avoiding areas of ENPP1 inactivating mutations present in GACI patients. These strategies allowed us to prioritize several dozen N-GCS sites for high throughput screening, which identified N-GCS sites in every ENPP1 protein domain designed to shield the ENPP1 protein surface. We found that the introduction of N-GCS reduced either the protein expression or the catalytic activity of most of these ENPP1 glycoforms with the substantial benefit only observed in one instance, the I256T mutation in clone 7. Interestingly, this N-GCS was an outlier not designed to cover the surface of the ENPP1 protein, instead the glycan is present in the ENPP3 insertion loop near the catalytic residue responsible for the nucleophilic attack of the catalyst on the substrate. The introduction of this site into ENPP1 increased AUC by approximately eightfold, primarily by increasing C_max after subcutaneous dosing, presumably by enhancing the absorbance of the subcutaneous bolus into the blood (Figure c1 and Figure 2b).

In our second strategy, based on protein engineering, we optimized FcRn recycling of the Fc domain; in contrast to the glycoengineering strategy, this method enhanced PK by increasing serum half-life with little effect on C_max. Quantitatively comparing the two techniques, adding a glycan at position 254 had a greater impact than Fc optimization, increasing AUC by ~ 8-fold compared with an ~ 6-fold increase from MST Fc mutations (clones 7 and 14 vs. 770; Figure 2a). Using both techniques in combination increased AUC 10-fold above the parent 770.

Figure 4 aSummary of the protein engineering steps. (a) Half-life and (b) area under the curve (AUC).
Glyco-engineering and protein-engineering represented by clone 17, usually applied separately unlike our combined approach, constitute the current limits of “upstream” efforts to improve therapeutic proteins. In this study, we reasoned that additional benefits could be obtained by implementing downstream biomanufacturing advances. We experimentally demonstrated this premise by expressing ENPPI-Fc with the combined I256T and MST mutations in α-2,6-ST overexpressing CHO cells and growing the cultures in the sialic acid precursor 1,3,4-O-Bu₃ManNAc; these 2 steps increased the AUC an additional 3-fold, resulting in a biologic with an ~13-fold increase over the parent biologic (Clone 19(ST) A vs. 770; Figure 3b), demonstrating the importance of glyco-polishing for improving PK properties. The PD effect of these changes was substantial; the increase in potency of the optimized therapeutic, as judged by normalization of plasma PPI, was ~37-fold (i.e., whereas construct 770 required 7.5 mg/kg to normalize plasma PPI for 7 days, 0.3 mg/kg of construct 19(ST) was able to normalize plasma PPI for 10.4 days). The overall strategy with a step-by-step illustration in PK improvements is provided in Figure 4.

Cost of goods, dosing frequency, and complications associated with immune reactions present barriers to the development of enzyme replacement therapy, and reducing dose levels and frequencies address critical commercial and clinical barriers to the development of these products. Our studies demonstrate that integrating protein design with techniques that enhance biologic PK enables the rational optimization of biologic enzymes to overcome these problems. Although the results must be reproduced in primates to determine their applicability to humans, we note that the murine half-life of our best construct is comparable to the primate half-life of a biologic, which was predictive of the optimized constructs, based on our murine data. Less frequent dosing would be clinically preferable for the chronic therapy required in GACI and ARHR2, as well as better tolerated in other diseases of vascular and soft tissue calcification induced by low PPI such as pseudoxanthoma elasticum and chronic kidney disease bone mineralization disorder, which may also respond to ENPPI-Fc therapy.

Supporting Information. Supplementary information accompanies this paper on the Clinical and Translational Science website (www.cts-journal.com).

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