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Genetically engineering transferrin to improve its *in vitro* ability to deliver cytotoxins

Dennis J. Yoon¹, David S.H. Chu¹, Christopher W. Ng¹, Edward A. Pham¹, Anne B. Mason², David Hudson³, Valerie C. Smith³, Ross T.A. MacGillivray³, and Daniel T. Kamei^{1,*}

¹ Department of Bioengineering, University of California, Los Angeles, CA 90095 U.S.A

² Department of Biochemistry, University of Vermont College of Medicine, Burlington, VT 05405 U.S.A

³ Department of Biochemistry and Molecular Biology and Centre for Blood Research, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

Abstract

We previously demonstrated that decreasing the iron release rate of transferrin (Tf), by replacing the synergistic anion carbonate with oxalate, increases its *in vitro* drug carrier efficacy in HeLa cells. In the current work, the utility of this strategy has been further explored by generating two Tf mutants, K206E/R632A Tf and K206E/K534A Tf, exhibiting different degrees of iron release inhibition. The intracellular trafficking behavior of these Tf mutants has been assessed by measuring their association with HeLa cells. Compared to native Tf, the cellular association of K206E/R632A Tf and K206E/K534A Tf increased by 126 and 250%, respectively. Surface plasmon resonance studies clearly indicate that this increase in cellular association is due to a decrease in the iron release rate and not to differences in binding affinity of the mutants to the Tf receptor (TfR). Diphtheria toxin (DT) conjugates of K206E/R632A Tf and K206E/K534A Tf showed significantly increased cytotoxicity against HeLa cells with IC₅₀ values of 1.00 pM and 0.93 pM, respectively, compared to a value of 1.73 pM for the native Tf conjugate. Besides further validating our strategy of inhibiting iron release, these Tf mutants provide proof-of-principle that site-directed mutagenesis offers an alternative method for improving the drug carrier efficacy of Tf.

Keywords

Transferrin; Diphtheria toxin; Site-directed mutagenesis; Intracellular trafficking; Targeted cancer therapy

1. Introduction

Human serum transferrin (Tf) is a monomeric glycoprotein (~ 80 kDa) involved in the transport of iron throughout the body. Each Tf molecule is capable of binding and delivering two Fe³⁺ (ferric) ions into cells. Iron delivery utilizes a receptor-mediated endocytic pathway via the transferrin receptor (TfR); the ferric ions play a key role in cellular growth and proliferation [1–3]. Therefore, TfR is expressed in virtually all nucleated cells of the body, with expression

*Corresponding Author: Daniel T. Kamei, 5121 Engineering V, University of California, Los Angeles, CA 90095, Tel: (310) 206-4826, Fax: (310) 794-5956, E-mail: E-mail: kamei@seas.ucla.edu.

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levels related to the iron requirements and proliferative status. Predictably, many studies have shown that TfR is overexpressed in a broad range of cancer cells as the result of their increased growth and proliferative capacity [2,3]. Investigations targeting the TfR thus provide a potentially important and novel approach to limiting cell proliferation.

While effective in combating cancerous cells, current radiation treatments and chemotherapies are marred by nonspecific toxicity. Thus, a compound capable of selectively targeting cancer cells might help alleviate the nonspecific toxicity associated with conventional therapeutics [4,5]. Exploiting the overexpression of TfR observed in many cancers, various ligands that bind TfR have been used to target therapeutics to cancer cells, including Tf, TfR monoclonal antibodies (mAbs) and Tf oligomers [6–16]. Although the use of Tf as a carrier of cytotoxins has been examined, the rapid recycling of Tf through the endocytic TfR pathway may significantly hinder its efficacy as a drug carrier. For instance, Tf trafficking studies performed on K562 cells (a human erythroleukemia-derived cell line) demonstrated the entire Tf cycle lasts only 4 to 5 minutes, while 30 such successive cycles of Tf-toxin conjugate trafficking may be required before the toxin's effect on the cell is observed [17,18]. Furthermore, once iron-bound Tf (holo-Tf) enters the trafficking pathway, it delivers its iron load to the cell, and iron-free Tf (apo-Tf) recycles to the cell surface. At the cell surface (pH ~ 7.4), apo-Tf has little to no binding affinity for TfR and is unable to re-enter the trafficking pathway until it binds more iron (Figure 1A) [19]. Rebinding of iron by Tf is a variable and inefficient process, and in many models of Tf trafficking, recycled Tf is often assumed to be lost [20,21]. Consequently, each iron-bound Tf molecule is restricted to a single 4 to 5 minute passage through the cell, making it difficult for a Tf-toxin conjugate to deliver its drug and achieve its intended purpose.

In order to increase the probability of delivering toxins to TfR expressing cancer cells, we wanted to increase the time Tf remained within a cell, *i.e.* its cellular association. To identify a design criterion for achieving this goal, we previously developed a mathematical model for the Tf/TfR cellular trafficking pathway using the principles of mass action kinetics [22,23]. A sensitivity analysis of the various parameters of this model identified decreasing the iron release rate in the endosome as a previously unreported design criterion. To understand this prediction, the Tf/TfR trafficking pathway must be re-considered. Briefly, once holo-Tf enters the cellular endosome, the acidification of the endosomal microenvironment (pH ~ 5.6) triggers the release of the two ferric ions, which are then transported out of the endosome. By preventing iron release in this acidified endosomal compartment, holo-Tf, instead of apo-Tf, returns to the cell surface. Since holo-Tf, unlike apo-Tf, retains its high affinity for TfR at a cell-surface pH of 7.4, it exhibits a slow dissociation rate. Hence, holo-Tf can either remain bound to TfR or quickly rebound another TfR if dissociation does occur, allowing holo-Tf to re-enter the cellular trafficking pathway (Figure 1B). Accordingly, cellular association is increased by increasing the number of cycles, not by lengthening the residence time of one cycle.

This prediction was subsequently confirmed by observing the changes in cellular association when the synergistic anion bicarbonate, involved in the loading of ferric ions onto Tf, was replaced with oxalate. The loading of a ferric ion onto each iron binding lobe of Tf typically involves two steps. First, contact binding of ferric ion to its binding site on Tf creates four coordination bonds to specific amino acid residues at that site. Second, under physiological conditions, the bicarbonate anion creates two more coordination bonds to the ferric ion, effectively locking the ferric ion in place [24]. Oxalate, with its lower pKa and symmetric conformation, binds more tightly to the ferric ion and significantly lowers the iron release rate of Tf [25]. Therefore, when we performed our cellular trafficking experiments, the cellular association of the oxalate Tf with HeLa cells was significantly greater than that of native Tf (Tf iron loaded using the naturally occurring bicarbonate anion). Additionally, we demonstrated that this increase in cellular association translated into an increase in potency

when DT conjugates of oxalate Tf were compared with DT conjugates of native Tf in an *in vitro* cell toxicity assay [22].

While the replacement of the synergistic anion, bicarbonate, with oxalate provides a simple and inexpensive means of improving the efficacy of Tf as a drug carrier, the current study was conducted to further explore the applicability of our general approach by generating two Tf mutants via site-directed mutagenesis that exhibited varying degrees of iron release inhibition. Apart from the amino acid residues involved in the direct binding of ferric ions to Tf, neighboring residues specifically trigger the release of iron in the acidic environment of the endosome [26–28]. In the N-terminal lobe (N-lobe) of Tf, K206 and K296 influence iron release, while in the C-terminal lobe (C-lobe), K534 and R632 are key residues affecting iron release. Mutations of these residues significantly lowered the iron release rate of Tf, and cellular trafficking studies with the mutants further validated our approach of inhibiting iron release to increase cellular association. Surface plasmon resonance (SPR) studies were also performed to confirm that the differences in cellular association were due to differences in iron release rates and not binding affinity for TfR. Furthermore, our results from MTT cell proliferation assays involving these mutants conjugated to DT confirmed that this increase in cellular association translates into an increase in cytotoxicity.

2. Materials and methods

2.1. Cell culture

HeLa cells (American Type Culture Collection, Manassas, VA) were seeded onto 75 cm² flasks (Corning Incorporated, Corning, NY) and proliferated in Minimum Essential Medium (Invitrogen, Carlsbad, CA) with 2.2 g/L sodium bicarbonate, 10% fetal bovine serum (Hyclone, Logan, UT), 1 mM sodium pyruvate, 100 units/mL penicillin (Invitrogen) and 100 µg/mL streptomycin (Invitrogen) at a pH of 7.4 (growth medium), and incubated in a humidified atmosphere with 5% CO₂ at 37°C. All reagents and materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Production of expression vectors for Tf mutants with lowered iron release kinetics

The Tf mutants were generated using site-directed mutagenesis as described previously [26, 27]. First, the N-terminus hexa-histidine tagged, nonglycosylated N-lobe mutant pNUT N-His K206E hTf NG expression vector was created. A polymerase chain reaction (PCR) based mutagenesis procedure [29] was used to introduce the mutation. The PCR products containing the mutation were ligated into Bluescript SK containing Tf/2N (a Tf variant containing two N-lobes) cDNA to determine the nucleotide sequences of the inserts confirming the introduction of the K206E mutation and the absence of others. The mutated Tf/2N cDNAs were excised from Bluescript SK, blunt-ended, and ligated into the *Sma*I site of the pNUT expression vector (pNUT K206E Tf/2N) followed by restriction endonuclease mapping to confirm correct orientation [26]. Next, an *Eco*RI-*Bam*HI DNA fragment from pNUT K206E Tf/2N was ligated with the *Eco*RI-*Eco*RI and *Eco*RI-*Bam*HI fragments from Tf-NG (nonglycosylated transferrin) in a triple ligation reaction creating pNUT N-His K206E hTf NG [27]. Restriction endonuclease mapping and DNA sequence analysis were used to confirm correct fragment orientation.

Next, the C-lobe mutations (K534A and R632A) were introduced into the pNUT N-His K206E hTf NG construct using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) [27]. The plasmid was initially denatured, and two sets of complimentary mutagenic oligonucleotide primers containing the mutations were annealed to their target sites. A PCR reaction was then performed to extend and incorporate the mutagenic primers, followed by digestion of the methylated, nonmutated parental DNA template. DNA sequencing was used to determine the presence of the correct mutation and absence of other mutations. This method was employed

to generate the mutant Tf expression vectors pNUT N-His K206E/K534A hTf NG and pNUT N-His K206E/R632A hTf NG. These expression vectors coded for two Tf mutants with changes to both iron binding lobes.

The expression vector pNUT N-His hTf NG was also created to code for the nonmutated N-terminus His-tagged nonglycosylated recombinant Tf (native Tf) as a control for the two Tf mutants [30].

2.3. Production and purification of recombinant proteins

The recombinant Tf plasmids were introduced into baby hamster kidney (BHK) cells for protein expression, and the recombinant protein was purified from the cell culture medium [30]. Briefly, the His-tagged protein was isolated by chromatography on a Ni-NTA column (Qiagen Inc., Valencia, CA) using a BioCad Sprint chromatography system to simultaneously monitor the absorbance at 280 nm, the conductivity, and the pH. Subsequently, a Sephacryl S200HR column was used to eliminate imidazole and exchange the protein into 0.1 M NH_4HCO_3 . Holo-Tf samples were exchanged into 50 mM HEPES buffer (pH 7.4) using Centricon YM-30 microconcentrators (Millipore, Billerica, MA) and stored at -20°C as concentrated stock solutions (~ 2.5 mM). At various stages of production and purification, the amount of recombinant Tf was determined by an adaptation of a competitive solid-phase radioimmunoassay in which biotinylated Tf was substituted for iodinated sample [31].

2.4. Radiolabeling Tf ligands

Tyrosine residues of holo-Tf ligands were radiolabeled with Na^{125}I purchased from MP Biomedicals (Irvine, CA) using IODO-BEADS (Pierce Biotechnology, Rockford, IL). Radiolabeled Tf ligands were then purified by size exclusion chromatography through a Sephadex G-10 column in the presence of bovine serum albumin to prohibit non-specific binding. The specific activity and concentration of each radiolabeled sample was determined by a phosphotungstic acid (PTA) assay.

2.5. Cellular trafficking of Tf ligands

The trafficking experiments were performed as described previously [22]. HeLa cells were seeded onto 35 mm dishes (Becton Dickinson and Company, Franklin Lakes, NJ) at a density of 3×10^4 cells/cm² in growth medium. After a 14 to 16 h incubation period in a humidified 5% CO_2 , 37°C environment, the growth medium was aspirated, and incubation medium containing 1 nM of radiolabeled Tf was added to each dish. The incubation medium was comprised of Minimum Essential Medium (MEM) supplemented with 20 mM HEPES, 1 mM sodium pyruvate, 100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (pH 7.4). The cells were then incubated at 37°C for 5, 15, 30, 60, 90, and 120 min, after which the incubation medium was removed, and the cells were washed five times with ice-cold WHIPS (20 mM HEPES, 1 mg/mL polyvinylpyrrolidone (PVP), 130 mM NaCl, 5 mM KCl, 0.5 mM MgCl_2 , and 1 mM CaCl_2 , pH 7.4) to remove nonspecifically bound ligand. To separate specifically bound Tf from internalized Tf, 1 mL of ice-cold acid strip (50 mM glycine-HCl, 100 mM NaCl, 1 mg/mL PVP, and 2 M urea, pH 3.0) was added to each dish and placed on ice for 8 min. Each dish was washed once more with 1 mL acid strip. The cells were solubilized by adding 1 mL of a 1 N NaOH solution to each dish for 30 min, followed by another 1 mL NaOH wash. The two basic washes were collected, and the solution was assayed for radioactivity using a Cobra Series Auto-Gamma Counter (Packard Instrument Co., Meriden, CT) to determine the amount of internalized ligand. The entire protocol, which had three replicates per time point per Tf ligand, was performed four times.

2.6. Surface plasmon resonance

SPR experiments were performed on a Biacore 3000 instrument (Uppsala, Sweden). For these experiments, a soluble form of TfR (sTfR) was immobilized on a CM5 sensor chip (Biacore) in random orientation using amine coupling chemistry. The carboxyl groups of the dextran surface were activated with *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (1:1). The sTfR (20 µg/mL in 5 mM maleate, pH 6.0) was applied to this activated surface until sufficient coupling was achieved (~ 1000 RUs in random orientation). Finally, a blocking solution (ethanolamine) was applied to the coupled surface to quench the remaining activated sites. Prior to Tf ligand binding, flow cells were equilibrated with running buffer (HEPES buffered saline (HBS) with 0.005% v/v surfactant P20, pH 7.4) until the baseline stabilized to less than 0.05 RU/min. Tf ligand binding experiments were performed at 24°C over a concentration series typically spanning a 10-fold range above and below the equilibrium dissociation constant (K_D) of the interaction. All SPR experiments were carried out in duplicate. The Tf-TfR interaction was monitored in running buffer with a flow rate of 40 µL/min, as previously described [32]. Data collection for the association phase and dissociation phase was 6 min each. A regeneration phase of 500 mM MgCl₂ (1 min) followed each cycle to return the response signal to baseline.

BIAevaluation v4.1 (Biacore) and the Scrubber software package (BioLogic Software, Campbell, Australia) were used to preprocess raw sensorgram data and make reference corrections for nonspecific binding, bulk refractive index changes, and background response from buffer-only injections. K_D values were determined from the above data using global, steady-state analysis fitting to a two-site binding model, according to the relationship: $R_{eq} = R_{max1}C/(K_{D1}+C) + R_{max2}C/(K_{D2}+C)$, where the subscripts 1 and 2 represent the first and second binding sites, respectively. R_{max} , R_{max1} and R_{max2} are the response signals at saturation, K_D , K_{D1} and K_{D2} are the equilibrium dissociation constants, and C corresponds to the injected analyte concentration.

2.7. Conjugation of Tf ligands with diphtheria toxin

DT conjugates of Tf ligands were prepared using 2-iminothiolane and sulfo-SMCC (Pierce) as crosslinkers to create a thioether bond [22]. DT in borate buffer (0.1 M sodium borate, 5 mM EDTA, 0.15 M NaCl, pH 8) was thiolated with a 0.625 mg/mL 2-iminothiolane-HCl solution (1:10) for 90 min at room temperature under nitrogen gas. The thiolated DT was separated from free iminothiolane by size exclusion chromatography using Zeba desalt spin columns (Pierce). Tf ligand in phosphate buffered saline (PBS; 50 mM sodium phosphate, 0.15 M NaCl, pH 7.4) was reacted with a 5 mg/mL sulfo-SMCC solution (1:6) for 60 min at room temperature. The modified Tf-SMCC compound was then separated from free sulfo-SMCC by size exclusion chromatography using the Zeba spin columns. The two modified protein solutions were diluted, incubated together overnight at 4°C under nitrogen gas and reduced in volume using Centrprep YM-10 centrifugal concentrators (Millipore). The DT conjugates with a 1:1 Tf:DT ratio in the concentrated solution were purified by HPLC (AKTA FPLC Chromatographic System, GE Healthcare Bio-Sciences, Piscataway, NJ) using two HiPrep 16/60 Sephacryl S-200 HR size exclusion columns in series (GE Healthcare Bio-Sciences); the identity of each peak was confirmed by SDS-PAGE. The concentration of the 1:1 Tf:DT conjugates was quantified using the Bradford dye binding assay.

2.8. Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay was used to quantify cell survival (Chemicon International, Temecula, CA) [22]. HeLa cells (1×10^4 cells/cm²) were seeded into each well of a 24-well tissue culture plate. After incubating the cells overnight, the growth medium was aspirated and incubated for 48 h with 450 µL of fresh growth medium containing varying concentrations of DT conjugates. Reagent

AB (45 μ L of 5 mg/mL MTT in PBS) was then added to each well and incubated for 4 h, followed by addition of 450 μ L of Reagent C (isopropanol with 0.04 M HCl) for color development. Visible light absorbance of each well was measured at 560 nm and 630 nm with an Infinite F200 plate reader (Tecan Systems Inc., San Jose, CA). The survival of cells relative to the control (cells incubated with growth medium containing no DT conjugates) was calculated by taking the ratio of the ($A_{560} - A_{630}$) values. This entire protocol, with four replicates per concentration per conjugate, was performed four times.

3. Results

3.1. Lowering Tf iron release rate through amino acid mutations of the N-lobe and C-lobe

In the current study, we examined an alternative means of manipulating the iron release rate of Tf by constructing Tf ligands, which had mutations of residues involved in the release of iron. In the Tf N-lobe, a lysine at position 206 was replaced by glutamate (K206E). In the Tf C-lobe, a lysine at position 534 or an arginine at position 632 was replaced by alanine (K534A or R632A). We generated two double Tf mutants, each with the K206E mutation in the N-lobe and either the K534A or R632A mutation in the C-lobe. Both had hexa His tags at the amino terminus and were nonglycosylated [27]. A summary of the iron release rates of these Tf mutants and N-His hTf NG (native Tf) is presented in Table 1.

3.2. Trafficking of Tf proteins in HeLa cells shows an increased cellular association of the Tf mutants compared to native Tf

We previously established that lowering the iron release rate of Tf translates into an increase in cellular association when the synergistic anion, carbonate, is replaced with oxalate. To further validate and generalize this strategy, *in vitro* cellular trafficking experiments in HeLa cells were conducted with the Tf mutants. As the metric for cellular association, the cumulative exposure of HeLa cells to Tf ligand was calculated as described previously [22]. Specifically, the area under the curve (AUC) of the internalized Tf values plotted as a function of time was determined and used as the metric. The results of the intracellular trafficking experiments show that HeLa cells internalized significantly greater amounts of the Tf mutants compared to their native counterpart (Figure 2). At the end of the 2 h incubation period, the internalization level of native Tf had reached 1.5×10^5 molecules/cell, compared to values of 4.5×10^5 and 6.4×10^5 molecules/cell for K206E/R632A Tf and K206E/K534A Tf, respectively. Comparing the AUC values of the 3 curves, we obtain a value of 1.6×10^7 , 3.6×10^7 and 5.1×10^7 (#·min)/cell for native Tf, K206E/R632A Tf and K206E/K534A Tf, respectively. These values translate into an AUC increase of 129% and 231% compared to the value for native Tf. These results were confirmed by three additional experiments yielding an average AUC increase of 126% and 250% for K206E/R632A Tf and K206E/K534A Tf, respectively.

3.3. Surface plasmon resonance analysis indicates that the increase in cellular association is only a function of decreased iron release kinetics

Although the mutations of the Tf protein are limited to the residues involved in the iron release mechanism, it is possible that these mutations could also affect the cell-surface binding of Tf to TfR, which would influence the interpretation of the cellular association results. To confirm that the mutations only affect the iron release rate of Tf and not its binding affinity for TfR, SPR was used to obtain equilibrium dissociation constant (K_D) values of our control and mutant Tf proteins at the cell-surface pH of 7.4. Binding isotherms were generated from equilibrium analysis of sensorgrams using the Scrubber software package. A bivalent-ligand model was utilized for this analysis, since each sTfR molecule exists as a dimer with two Tf binding sites. Similar binding response patterns were observed for all Tf proteins (Figure 3), and equilibrium binding analysis was used to verify the equilibrium dissociation constants of each mutant to the sTfR, yielding two such values (K_{D1} and K_{D2}) for each binding site of TfR.

Random orientation immobilization was selected to immobilize sTfR to the CM5 sensor chip for its ease and convenience. However, an artifact of the random orientation amine coupling strategy is the potential occlusion of one of the sTfR dimer binding sites during immobilization. In this situation, only one binding site of the dimer is free to bind Tf. Therefore, K_{D1} is unaffected by immobilization, while an unknown amount of the second binding site can be largely blocked, causing errors in K_{D2} . As such, with random orientation immobilization, the bivalent model is used to fit the data, but the K_{D2} is simply used to provide a better fit. Note that this immobilization strategy has been successfully employed in the past for analyzing binding to TfR [32,33], creating the precedent for evaluating only the first K_D in this bivalent-ligand system.

As seen in Table 2, the primary equilibrium dissociation constant (K_{D1}) values calculated from the equilibrium binding response isotherms were 1.54 nM for native Tf, 0.93 nM for K206E/R632A Tf and 1.12 nM for K206E/K534A Tf. Although a t-test analysis of the K_D values showed one statistically significant pairwise difference (native Tf K_D vs. K206E/R632A Tf, $p = 0.013$), all three K_D values are quite similar and lie within the published acceptable range for the Tf-TfR binding interaction [32,34]. As a result, the manipulation of the Tf iron release rate seems to be the major contributing factor to the increased cellular association observed for the Tf mutants.

3.4 Diphtheria toxin conjugates of Tf mutants exhibit increased HeLa cell toxicity over native Tf

After confirming that the increased cellular association of the two Tf mutants was a result of inhibiting iron release, we tested their drug carrier efficacy. As in our previous oxalate studies [22] we developed DT conjugates of our Tf proteins, and administered them to HeLa cells over a range of DT concentrations (10^{-5} nM to 10^{-2} nM). The inhibition of cellular growth was then assessed after a 48 h incubation period using the MTT assay. Similar to the effect of oxalate Tf, the two dual lobe Tf mutants showed a significantly greater drug carrier efficacy relative to native Tf (Figure 4). IC_{50} values, the concentrations at which 50% inhibition of cellular growth occurs, were 1.79 pM for native Tf compared to 0.93 pM and 1.00 pM for K206E/K534A Tf and K206E/R632A Tf, respectively.

4. Discussion

Due to its increased expression in a variety of tumor cells, TfR has been evaluated as a target for the selective delivery of cytotoxic agents to tumor cells. Tf has been conjugated to cytotoxic agents (e.g. doxorubicin, diphtheria toxin, ricin and gelonin) to study their ability to target these drugs to tumor cells, while reducing their nonspecific toxic effects on non-tumor cells [5,8]. However, the rapid recycling rate of Tf may significantly reduce its efficacy as a drug carrier. For instance, studies of the Tf-gelonin conjugate have suggested that for every 10 million conjugates that are recycled, only one molecule of gelonin is actually delivered to the cell [7]. Furthermore, while the conjugates are initially produced with holo-Tf, which has a very high affinity for TfR, once the conjugate enters the Tf-TfR endocytic pathway, the Tf delivers its iron load to the cell, and leaves the cell as apo-Tf, which has little or no affinity for TfR at physiological pH. Thus, if the conjugate cannot deliver its load during its first cycle through the cell, the Tf conjugate is lost until it acquires more iron in an inefficient process [20,21]. In healthy individuals, ~30% of the circulating Tf is iron saturated [35].

While other researchers have used Tf mAbs and Tf oligomers to address the inefficiency of Tf as a drug carrier, we have been focusing on engineering Tf itself. We previously demonstrated that by reducing the iron release rate of Tf in the endosomal compartment of the trafficking pathway, the probability of holo-Tf, rather than apo-Tf, returning to the cell surface would increase, making it available to quickly re-enter the cell via receptor-mediated endocytosis

[22]. With regard to drug delivery, this would increase the association of a Tf-toxin conjugate with a cell, which, in turn, would increase the likelihood of a Tf-toxin conjugate delivering its toxic payload to the cell.

In our previous work, we engineered Tf by replacing the Tf synergistic anion, carbonate, with oxalate. The current study, on the other hand, focused on further validating our approach of engineering Tf to slow or completely prevent iron release. Specifically, Tf mutants were generated to demonstrate proof-of-principle that site-directed mutagenesis provides an alternative method to replacing the synergistic anion for improving the drug carrier efficacy of Tf. To engineer the Tf variants, a single amino acid in each lobe of Tf was mutated. In the Tf N-lobe, iron release is driven in part by residues K206 and K296 [26,36]. These residues reside in opposite domains of the iron binding cleft of the N-lobe, and in the iron loaded state at physiological pH, they share a hydrogen bond (suggesting an abnormally low pKa for one of the lysines). However, once the Tf-TfR complex is internalized and reaches the acidic environment of the endosome, both lysines become protonated. The two positively charged lysines then repel each other, driving the two domains apart, opening the iron binding cleft and facilitating iron release. Moreover, studies also revealed that mutating one of these lysines to glutamate (*i.e.* K206E) significantly inhibited iron removal from the N-lobe. Since the glutamate residue remains deprotonated in the endosome, the negative charge further stabilizes the attractive, electrostatic interaction across the iron binding cleft by forming a salt bridge. In addition, iron removal from the C-lobe is under the influence of residues K534 and R632 [27,28]. The mechanism of iron removal involving these residues is less understood. However, alanine scanning has revealed that substituting either the K534 or R632 residue with alanine significantly inhibits iron removal from the C-lobe [27].

We generated the mutants K206E/R632A Tf and K206E/K534A Tf, which exhibited lower iron release rates relative to native Tf (Table 1). We predicted that the K206E/R632A Tf and K206E/K534A Tf mutants would associate with HeLa cells to a greater extent compared to native Tf. As expected, the K206E/R632A Tf and K206E/K534A Tf mutants demonstrated an increase in cellular association with AUC values that were an average of 126% and 250% greater than native Tf, respectively. To demonstrate that these increases in cellular association were due to inhibiting iron release and not due to affecting binding of Tf for TfR, SPR analysis of ligand binding to sTfR was performed. SPR binding experiments conducted with the two Tf mutants and native Tf confirmed that the binding behavior of the ligands were similar with equilibrium dissociation constant values between 0.93 and 1.54 nM, well within the range reported in the literature for the native Tf/TfR interaction [34]. Thus, the mutations to Tf increased the cellular association of the Tf mutants with HeLa cells by reducing its iron release rate and not by increasing its binding affinity for TfR.

Though, in general, a decrease in iron release rate leads to an increase in cellular association, the cellular association does not precisely correlate with the decreased iron release rate. Thus, even though K206E/K534A Tf has a 3.5-fold higher iron release rate in its C-lobe than K206E/R632A Tf (Table 1), it outperformed K206E/R632A Tf in terms of cellular association by 1.5-fold. This discrepancy was a result of the iron release data being determined *in vitro in the absence of TfR*. An investigation by Gianetti *et al.* [33] explored the role of TfR in stimulating the release of iron from Tf in the endosomal compartment. It was concluded from this study that the Tf/TfR interaction within the endosomal compartment is essential for the rapid release (~ 2–3 minutes) and delivery of iron to the cell [33,37]. This ligand-receptor complex, in the acidic environment of the endosome, stabilizes the open form of Tf. In doing so, the coordination bonds between Tf and the ferric ions are disrupted, allowing Tf to release its iron to the cell. When comparing the iron release rate at pH 5.6 of isolated holo-Tf to holo-Tf bound to TfR, it was determined that the presence of TfR enhanced the iron release rate of holo-Tf ~104-fold. Accordingly, the iron release data in Table 1 provide a first approximation for the

effects of the Tf mutations on iron release rate. Moreover, since *in vitro* experiments with sTfR can never truly replicate the exact environment of the endosome, cellular trafficking experiments are critical to accurately evaluate the effects of these mutations on cellular association.

Similar to our previous oxalate studies, we produced conjugates of the Tf mutants with the DT cytotoxin to determine if the increases observed in HeLa cell association translate to an increase in drug carrier efficacy. Results show that indeed the Tf mutant conjugates display an increased toxicity toward HeLa cells compared to native Tf. The IC₅₀ values for K206E/K534A Tf and K206E/R632A Tf were 0.93 pM and 1.00 pM, respectively, compared to a value of 1.79 pM for native Tf. These Tf mutants, as in the case of oxalate Tf, provide an effective means of increasing the drug carrier efficacy of Tf through a reduction in iron release kinetics. It is also encouraging to find that the order of increasing cellular association exhibited by the Tf mutants (K206E/K534A Tf > K206E/R632A Tf > native Tf) is preserved in the order of increasing cellular toxicity when conjugated to DT. Accordingly, we have further validated our approach of engineering Tf by inhibiting iron release, and these Tf mutants generated with site-directed mutagenesis have potential in enhancing the drug delivery potential of Tf.

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Figure 1.
 (A) Schematic of the Tf/TfR trafficking pathway. (B) The proposed alternative Tf/TfR trafficking pathway

Figure 2. Representative results of internalized Tf/cell as a function of time for the two Tf mutants and native Tf at a 1 nM concentration. Error bars represent the standard deviation from an average of three measurements.

Figure 3. SPR analysis of Tf binding to immobilized sTfR. (A–C) Binding profiles of native Tf, K206E/R632A Tf, and K206E/K534A Tf, respectively, at various concentrations. (D–F) Plots of equilibrium binding response vs. concentration of injected protein, derived from biosensor experiments for native Tf, K206E/R632A Tf, and K206E/K534A Tf, respectively.

Figure 4. Cytotoxicity comparisons for DT conjugates made with native Tf, K206E/K534A Tf and K206E/R632A Tf. Error bars represent the standard error from an average of four experiments. (A) Native Tf vs. K206E/R632A Tf. (B) Native Tf vs. K206E/K534A Tf. 95% confidence intervals for the best-fit curves are represented by the faint lines.

Table 1

Iron release rates of the various Tf variants in the presence of 12 mM Tiron (pH 7.4) or 4 mM EDTA (pH 5.6) [27].

	pH 7.4, N-lobe (min ⁻¹)	pH 7.4, C-lobe (min ⁻¹)	pH 5.6, N-lobe (min ⁻¹)	pH 5.6, C-lobe (min ⁻¹)
Native Tf	0.044±0.002	0.037±0.002	16.8±1.3	0.139±0.006
K206E/R632A Tf	No release	No release	0.0006±0.00005	0.0015±0.00009
K206E/K534A Tf	No release	No release	0.00057±0.00004	0.0052±0.00005

Table 2

The measured primary equilibrium dissociation constants (K_{D1}) for all Tf ligands at pH 7.4.

Tf ligand	K_{D1} (nM)
Native Tf	1.54 ± 0.09
K206E/R632A Tf	0.93 ± 0.11
K206E/K534A Tf	1.12 ± 0.19