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Applying the Fe(III) Binding Property of a Chemical Transferrin Mimetic to Ti(IV) Anticancer Drug Design

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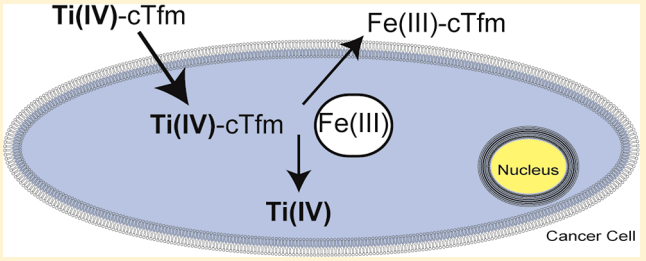
Supporting Information

ABSTRACT: As an endogenous serum protein binder of Ti(IV), transferrin (Tf) serves as an excellent vehicle to stabilize the hydrolysis prone metal ion and successfully transport it into cells. This transporting role is thought to be central to Ti(IV)'s anticancer function, but efforts to synthesize Ti(IV) compounds targeting transferrin have not produced a drug. Nonetheless, the Ti(IV) transferrin complex (Ti_2Tf) greatly informs on a new Ti(IV)-based anticancer drug design strategy. Ti_2Tf interferes with cellular uptake of Fe(III), which is particularly detrimental to cancer cells because of their higher requirement for iron. Ti(IV) compounds of chemical transferrin mimetic (cTfm) ligands were designed to facilitate Ti(IV) activity by attenuating Fe(III) intracellular levels. In having a higher affinity for Fe(III) than Ti(IV), these ligands feature the appropriate balance between stability and lability to effectively transport Ti(IV) into cancer cells, release Ti(IV) via displacement by Fe(III), and deplete the intracellular Fe(III) levels. The cTfm ligand *N,N′*-di(*o*-hydroxybenzyl)ethylenediamine-*N,N′*-diacetic acid (HBED) was selected to explore the feasibility of the design strategy. Kinetic studies on the Fe(III) displacement process revealed that Ti(IV) can be transported and released into cells by HBED on a physiologically relevant time scale. Cell viability studies using A549 cancerous and MRC5 normal human lung cells and testing the cytotoxicity of HBED and its Ti(IV), Fe(III), and Ga(III) compounds demonstrate the importance of Fe(III) depletion in the proposed drug design strategy and the specificity of the strategy for Ti(IV) activity. The readily derivatized cTfm ligands demonstrate great promise for improved Ti(IV) anticancer drugs.



Titanium(IV) compounds have long held the promise to complement or even supplant platinum-based anticancer drugs because of their broad spectrum of effect in vitro.[1−3] Their instability in aqueous solution, however, has greatly deterred this promise. This instability stems from their rapid dissociation into inactive hydrolyzed products, namely, titanium oxide species.[4] Transferrin (Tf), the iron(III) transport serum protein, is widely implicated in the anticancer properties of Ti(IV). The interaction of Tf with the unstable Ti(IV) compounds is thought to be crucial in rescuing their activity. As demonstrated with the former drug candidate titanocene dichloride and other Ti(IV) compounds, Tf can coordinate Ti(IV) at both of its homologous metal binding sites (Figure 1) with high affinity and at fast rates.[4,5] Ti(IV)-bound Tf enables the endocytotic delivery of Ti(IV) into cells through the Tf receptors. Tf would appear to be an excellent anticancer drug delivery agent for Ti(IV) because of the overexpression of Tf receptors in many cancer cells,[6−8] facilitating cancer cell receptor-mediated selectivity. Ti(IV)-saturated Tf (Ti_2Tf) interferes with Fe(III) endocytosis and has been observed to inhibit Fe(III)-saturated Tf (Fe_2Tf) from entering a placental cancer cell line.[8] Evidence points to Ti_2Tf serving as an effective anticancer drug.

Efforts to develop stable Ti(IV) compounds that can efficiently deliver Ti(IV) to Tf have proven unsuccessful. When examining the cytotoxic properties of these compounds, the half-maximal inhibitory concentrations (IC_{50}) of these compounds against different cell lines were negligibly effected by the presence of Tf.[9−13] Furthermore, two different formulations of Ti_2Tf showed no cytotoxicity against A549 lung cancer cells, but Ti(IV) in a small compound form did.[13] The insignificant impact of Tf on Ti(IV) cytotoxicity in these studies may be due to a poor Ti(IV) release mechanism from endosomal Tf[14] preventing Ti(IV) from attacking intracellular targets. These findings do not negate the importance of Tf and potentially other serum proteins such as albumin[15,16] in providing stability and intracellular transport for certain Ti(IV) compounds[17] but does indicate that Tf may not be as central to the metal's cytotoxicity as previously hypothesized.

While targeting Tf for Ti(IV) delivery may not produce an effective drug, the Ti(IV) Tf complex greatly informs on a new strategy for designing Ti(IV) anticancer compounds. A recent in vivo study showed that Ti(IV) is endogenously bound to Tf.[18] The Tf binding site essentially facilitates Ti(IV) bioavailability. It is likely a major contributor to the metal's high serum concentration (~2 μM)[19] relative to its ligand-free solubility. It thus provides insight into a valuable template for stably chelating and transporting Ti(IV). We hypothesized that this template can be weaponized for therapeutic application. In obstructing

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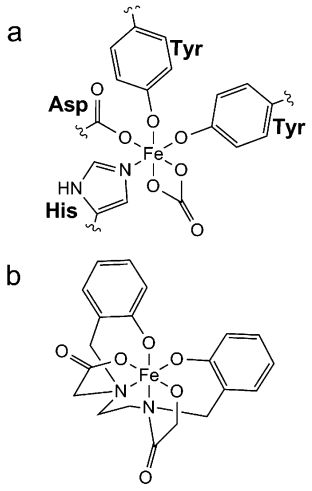

Figure 1. Fe(III) coordination by human serum transferrin (a) and HBED (b). Transferrin (Tf) metal binding site consists of two tyrosines, one histidine, and one aspartate and the synergistic anion carbonate. HBED models Tf's metal coordination and serves as a useful chemical transferrin mimetic.

receptor upload of Fe_2Tf, Ti_2Tf can disrupt Fe(III) biochemistry with potential to cause cell death.[8] Cancer cells have a higher requirement for iron than normal cells[20] and express higher levels of the transferrin receptor to meet demand. Decreasing intracellular levels of iron in cancer cells would be very detrimental to their survival. A drug design strategy focused on development of iron chelators for anticancer treatment has shown promise.[20,21] However, developing a synergistic drug that combines the antiproliferative property of Ti(IV) with the Fe(III)-depleting capacity of an iron chelator would be even more powerful.

A cell-permeable ligand that mimics the Tf metal binding site would provide not only the necessary chelation stability to transport Ti(IV) into cancer cells but also the coordination chemistry preference for binding Fe(III). Tf exhibits this preference as shown in a study where Fe(III) was able to displace Ti(IV) from Ti_2Tf.[8] A chemical Tf mimetic (cTfm) that possesses this binding preference for Fe(III) would interfere with intracellular levels of Fe(III). Such a ligand's higher affinity for Fe(III) would enable the thermodynamic drive for releasing Ti(IV) and deplete cells of iron. It is critical for this metal displacement process to occur on a time scale in which it can take place within cancer cells, allowing Ti(IV) to bind to intracellular targets, possibly DNA[22,23] or enzymes,[24−26] where it can exhibit its effect. In this drug design strategy, the cTfm ligands would serve as both passive Ti(IV) transport agents and active triggers for cytotoxicity due to their Fe(III) binding capacities (Figure 2).

The ligand *N,N′*-di(*o*-hydroxybenzyl)ethylene-diamine-*N,N′*-diacetic acid (HBED) (Figure 1) is an excellent candidate to investigate the feasibility of this drug design strategy. HBED produces metal compounds[13,14,27] with structural and spectral features comparable to the corollary transferrin compounds.[13,14] Once considered as a potential drug for iron-overload diseases,[28,29] HBED exhibits a very high affinity for Fe(III) (log K = 39.01).[27] This affinity is of the formulation of a monomeric species ($[FeHBED]^-$)[30] with HBED as a hexadentate and fully deprotonated ligand. Aqueous speciation studies indicate that at physiological pH HBED exhibits a greater affinity for Fe(III) than for Ti(IV) because of Ti(IV)'s higher propensity to undergo hydrolysis. HBED binds Ti(IV), in a

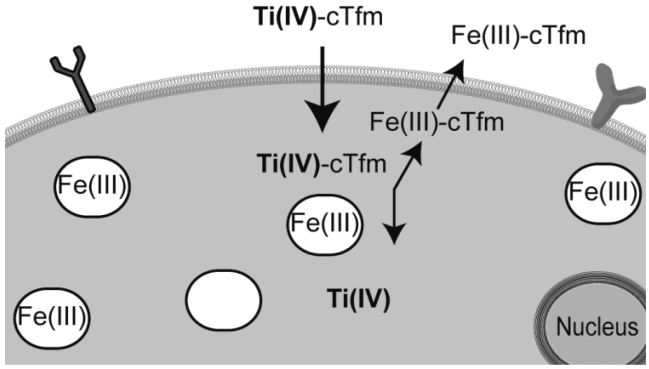

Figure 2. Drug design strategy for Ti(IV)-based anticancer compounds involving use of chemical transferrin mimetic (cTfm) ligands in the transport of Ti(IV) into cancer cells and release upon metal displacement by Fe(III). Combination of Fe(III) depletion by cTfm and Ti(IV) binding to intracellular targets triggers cytotoxicity.

titanyl unit form ($[TiOHBED]^-$),[13] as a pentadentate and monoprotonated ligand. Nonetheless, HBED coordinates Ti(IV) in solution as a stable and monomeric compound and transports Ti(IV) into cancer cells.

Herein, we investigate the contributions of the Fe(III) binding capacity of HBED to the cytotoxicity of $[TiOHBED]^-$ previously observed in A549 lung cancer cells[13] and extend it to a noncancer cell line (MRC5). While Fe(III) displacement of Ti(IV) from HBED coordination is thermodynamically favorable, it is necessary to determine if the kinetics occur on a biological time scale using a biorelevant source of Fe(III). To explore the hypothesis that HBED's Fe(III) depletion works in synergism with Ti(IV), the cytotoxicity of the Ti(IV) and Fe(III) compounds of HBED and of the metal-free ligand is studied against the A549 and MRC5 cell lines. The Fe(III) HBED compound should show little if any cytotoxicity because it is unable to coordinate additional Fe(III). The Ga(III) compound of HBED is also examined because Ga(III) is an Fe(III) biomimic.[31] Ultimately, $[TiOHBED]^-$ displays the strongest cytotoxicity against both cell lines. This work points to cTfm molecules as a family of ligands with the chemical and biological traits specifically tailored to facilitating Ti(IV) anticancer activity. It presents a very promising and bioinspired new design strategy, which manipulates ligand metal affinity for therapeutic effect.

MATERIALS AND METHODS

All aqueous solutions were prepared with nanopure-quality water (18.2 MΩ·cm resistivity; model Thermoscientific Easypure Rodi; Barnstead). *N,N′*-Di(*o*-hydroxybenzyl)ethylenediamine-*N,N′*-diacetic acid (HBED) was purchased from Strem Chemicals. TiHBED was synthesized as previously described.[13] $FeCl_3$ and $Ga(NO_3)_3$ were obtained from Sigma. All other chemicals were of high purity and used as received.

Synthesis of $Na[Fe(C_{20}H_{20}N_2O_6)]\cdot CH_3CH_2OH$. $FeCl_3$ (1 mmol) was dissolved in 10 mL of water and HBED (1 mmol) was dissolved in 10 mL of water. Solutions were mixed, and the dark red solution turned brighter red when the pH was raised to 7.0. The red solution was rotovapped to dryness. Crude product was suspended in dry ethanol to solubilize the desired product and eliminate any salts. The red solution that was obtained was centrifuged for 10 min at 14 000 rpm to rid of any insoluble precipitate. The final solution was rotovapped until dryness, producing a pure red product that is water soluble (29.6% yield). CHN elemental analysis was performed by Atlantic Microlabs (Norcross, GA) [data are displayed as found (calcd)]: C, 51.77 (51.88); H, 5.20 (5.15); N, 5.61 (5.50). UV−vis (H_2O) at pH 7.4: λ_{max} = 484 nm (ε = 3970 M^{-1} cm^{-1}). FT-IR data (cm^{-1}): $\nu_{as}(CO_2)$ 1635, 1596; $\nu_s(CO_2)$ 1481, 1451.

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Mass spectrum (negative-ion mode): $[Fe(C_{20}H_{20}N_2O_6)]^-$ m/z = 440.32. ^{1}H NMR (ppm): 7.468(t); 7.539(m); 7.858(m). All peaks are broad and shifted to lower field relative to the metal-free ligand: 6.994(m), 7.279(d), 7.343(d), 7.398(m).

Synthesis of Na[Ga($C_{20}H_{20}N_2O_6$)]. $Ga(NO_3)_3$ (1 mmol) was dissolved in 10 mL of water, and HBED (1 mmol) was dissolved in 10 mL of water. Solutions were mixed, and the pH of the colorless solution was raised to 7.0. The solution was rotovapped to dryness. Crude product was suspended in dry ethanol to solubilize the desired product and eliminate any salts. The colorless solution that was obtained was centrifuged for 10 min at 14 000 rpm to rid of any insoluble precipitate. The final solution was rotovapped until dryness, producing a pure off-white product that is moderately water soluble (31.4% yield). CHN elemental analysis found (calcd): C, 50.43 (50.35); H, 4.31 (4.23); N, 5.81 (5.87). FT-IR data (cm^{-1}): $\nu_{as}(CO_2)$ 1653; $\nu_s(CO_2)$ 1483, 1455. Mass spectrum (negative-ion mode): $[Ga(C_{20}H_{20}N_2O_6)]^-$ m/z = 454.21. ^{1}H NMR (ppm): 6.814(m); 7.078(d); 7.168(d); 7.253(m). All peaks are shifted to higher field relative to the metal-free ligand.

Instruments. A Cary 1E UV−vis spectrophotometer was used for kinetics experiments. All pH values were determined using a Thermo Scientific Orion Star A111 and an Orion 9157BNMD electrode, calibrated with buffer solutions at pH 4, 7, and 10. A Buchi rotovapor R-200 and 5702 Eppendorf centrifuge were used for all synthetic protocols. FT-IR spectra were collected on a Thermo Nicolet 470 spectrometer using KBr pellets. Mass spectra were collected on a Waters Micromass Q-Tof at a capillary voltage of 3000 and sample cone voltage of 30. ^{1}H NMR solution spectra were recorded on a Bruker 500 MHz instrument in D_2O.

Kinetics of Ti(IV) Displacement from HBED by Fe(III). $[TiOHBED]^-$ was reacted with Fe(III) citrate at pH 7.4 (0.1 M Tris buffer; 0.1 M NaCl). A stable formulation of Fe(III) citrate[32] was prepared in situ by mixing aqueous solutions of $FeCl_3$ and citric acid (held in at least a 10-fold excess relative to Fe(III) concentration). The mixture was diluted in buffer to desired concentrations. $[TiOHBED]^-$ was also prepared in situ by dissolving TiHBED in buffer and adjusting pH accordingly.[13] The concentration of $[TiOHBED]^-$ was maintained constant at 50 μM in all final reaction solutions. The kinetics was followed by monitoring formation of $[FeHBED]^-$ over the wavelength range from λ = 400 to 700 nm. The rate dependence of $[FeHBED]^-$ formation on Fe(III) and citrate^{3-} concentrations was investigated. In one set of experiments, Fe(III) concentrations ranged from 50 to 500 μM while the citrate concentration was maintained constant at 10 mM. In another set, the citrate concentrations ranged from 1 to 10 mM while the Fe(III) concentration was maintained at 50 μM. Reactions were measured at set time increments over 12 h, every minute for the first 20 min and then every 10 min for 12 h to determine the time scale of the reaction at which equilibrium is reached. Rates ($d[FeHBED]^-/dt$) at varying concentrations were measured by the method of initial rates from the first 20 min time frame, and rate data were fitted to eq 1 using the nonlinear iterative regression feature of Origin 7.0. All experiments were replicated four times. Error values are reported as standard deviations for all experiments.

For insight into the mechanism of this reaction, the reaction was followed by mass spectrometry (n = 4). All solutions were prepared in a volatile buffer consisting of 0.1 M $(NH_4)_2CO_3$, pH 7.4. A 2 mM $[TiOHBED]^-$ solution was mixed with a solution of Fe(III) citrate (2 mM Fe(III); 20 mM citrate) and immediately followed by direct infusion to the detector. The mass range was from 300 to 1000 m/z.

A549 and MRC5 Cell Viability Assays. A549 human lung cancer cells and MRC5 human lung normal cells were obtained from ATCC (CCL-185 and CCL-171, respectively). Both cell lines were cultured in phenol red-free DMEM (Cellgro) containing 10% FBS (HyClone) and penicillin/streptomycin (Calbiochem and EMD, respectively) at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 and incubated at these conditions for all steps of the cell viability study. Cells at >90% confluence [grown in 150 × 25 mm tissue-culture dishes (BD Falcon)] were seeded into 96-well plates in DMEM. Plates containing A549 cells were seeded with a volume of 100 μL at 5.6×10^4 cells per well on day 0. Plates containing MRC5 cells were seeded with a volume of 100 μL at 8.8×10^4 cells per well on day 0. Variation in quantities of cells for A549 and MRC5 was due to the varying growth rates. Cells were incubated for 24 h. They were then treated with $HBED^-$, $[TiOHBED]^-$, $[FeHBED]^-$, and $[GaHBED]^-$. Metal-bound and metal-free HBED solutions were prepared in pH 7.4 PBS buffer containing 0.13 M NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4. Stock solutions of $HBED^-$, $[TiOHBED]^-$, $[FeHBED]^-$, and $[GaHBED]^-$ were prepared in DMF and then diluted to specific concentrations (0.1 −200 μM) in PBS while maintaining DMF at 1.0% (v/v). All final buffered solutions were prepared immediately before addition to the cultured cells to limit the possibility of precipitation upon standing. Samples were added to the cells in a volume of 100 μL with four replicates per concentration. Plates were incubated for 3 days. At 3 h before completion of the incubation time, plates were prepared for colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. A 25 μL of MTT (Research Organics) solution (sterile filtered) with a concentration of 9.76 mg/mL in PBS buffer was added to each well. Plates were kept protected from light and then incubated for the remaining 3 h. A 50 μL amount of 24.42% (w/v) SDS solution (sterile-filtered) was then added to each well and incubated overnight. Absorbances of all plates were measured at 590 nm using a plate reader (Multiskan FC, Thermoscientific). IC_{50} values were determined from dose−response curves.

Fe(III) Rescue Cell Viability Experiment. A cell viability experiment was performed using the conditions above. After incubating A549 and MRC5 cells in the 96-well plates for 1 day, the media of some of the cells were supplemented with 100 μM Fe(III) citrate (100 μM Fe(III); 200 μM citrate) and cells were incubated for an additional 2 h. At this point, 100 μM of either $HBED^-$ or $[TiOHBED]^-$ was added to the cells, treated and untreated with Fe(III) citrate. Control cells were maintained to which no compounds were added. MTT assay was performed after an additional 3 day incubation. A student T-Test was performed to evaluate the differences in cell viability between treatment and no treatment with Fe(III) citrate.

RESULTS AND DISCUSSION

Establishing a Time Scale for Ti(IV) Release from HBED by Fe(III) Displacement. One major component of our proposed Ti(IV)-based anticancer drug design strategy is to carefully consider the metal coordination preference of the chemical Tf mimetics. The idea is to use these ligands as transport vehicles to directly release Ti(IV) in cancer cells via metal displacement so that it can attack intracellular sites. HBED as a representative of this family of ligands is an excellent case study for development of the drug design strategy. Our previous observation of the cytotoxic behavior of $[TiOHBED]^-$ against A549 lung cancer cells[13] bodes very well for this initiative. The complex was shown to successfully cross the cell membrane.[13] The intracellular environment is the site of activity of the design strategy. HBED has a stronger affinity for Fe(III) than it does Ti(IV) at pH 7.4 and even down to pH ≈ 5.8,[13,27] which is important because the intracellular environment of cancer cells tends to be more acidic than normal cells.[33] At physiological pH, displacement of Ti(IV) from HBED coordination by Fe(III) would be a thermodynamically favorable reaction.

We explored the kinetics of the metal displacement process under physiologically relevant conditions to determine whether this reaction is a feasible step in the anticancer mechanism of $[TiOHBED]^-$. To this end $[TiOHBED]^-$ was reacted with a source of Fe(III) at pH 7.4. Fe(III) was supplied in a biorelevant formulation as a citrate chelated compound[34] because in the body chelation by small molecules and proteins provides solubility for iron ions and prevents these ions from engaging in toxic free radical reactions.[35] Citrate is present at 100 μM concentration in human serum and serves as a nontransferrin-bound iron (NTBI) ligand.[36] Despite forming relatively labile complexes of Fe(III) and Ti(IV), citrate highly stabilizes both

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metal ions in solution.[32,34,37] In the experiment, citrate also served the function of chelating Ti(IV) displaced from HBED. At the concentrations of citrate used, Fe(III) and Ti(IV) exist as the $[Fe(C_6H_4O_7)_2]^{5-}$ and $[Ti(C_6H_4O_7)_3]^{8-}$ species.[32,34,37] Also under the experimental conditions, citrate and HBED exist as the $C_6H_5O_7^{3-}$ and $C_{20}H_{23}N_2O_6^-$ species,[27,38] respectively.

The kinetics study was performed using the method of initial rates. The metal displacement rate was measured by monitoring formation of $[FeHBED]^-$ using UV−vis spectroscopy in the 400−700 nm window where $[FeHBED]^-$ is the only absorbing species (λ_{max} = 484 nm; ε = 3970 M^{-1} cm^{-1}). The rate dependence on Fe(III) and citrate^{3-} concentrations was examined while maintaining the concentration of $[TiOHBED]^-$ constant (50 μM). This concentration of $[TiOHBED]^-$ was selected because it is in the micromolar range where $[TiOHBED]^-$ exhibits cytotoxicity (Table 1). There was a hyperbolic growth rate dependence on Fe(III) and citrate^{3-} concentrations (Figure 3), implicating the existence of an intermediate in the metal displacement process. A mass spectrometry experiment was performed to detect this transient intermediate because there was no distinctive UV−vis signal for it, probably owing to very little build up. A low-resolution quaternary complex of Fe(III), TiO^{2+}, HBED, and citrate was detected in negative-ion mode with the elemental formula for the postulated species $Na_2[TiO(HBED)Fe(Citrate)]^-$ ($Na_2[TiO(C_{20}H_{20}N_2O_6)Fe(C_6H_4O_7)]^-$; m/z = 738.20) in which sodium ions serve as counterions (Figure 4).

Table 1. Effects of Ti(IV), Fe(III), and Ga(III) HBED Compounds, Metal-Free HBED, and Other Ti(IV) Compounds on A549 and MRC5 Cell Viability (IC_{50}, μM)

agent	IC_{50} (μM) A_{549}	IC_{50} (μM) MRC_5
$[TiOHBED]^-$	24.1 ± 1.2[a]	42.0 ± 4.4
$[FeHBED]^-$	>100	≫100
$[GaHBED]^-$	>100	≫100
$HBED^-$	105.2 ± 13[a]	≫100
Ti citrate	≫100[b]	*c*
Ti(naphthalene-2,3-diolate)$_3^{2-}$	≫100[b]	*c*

[a]Compares favorably with previous measurement (ref 13). [b]Obtained from ref 13. [c]Not applicable.

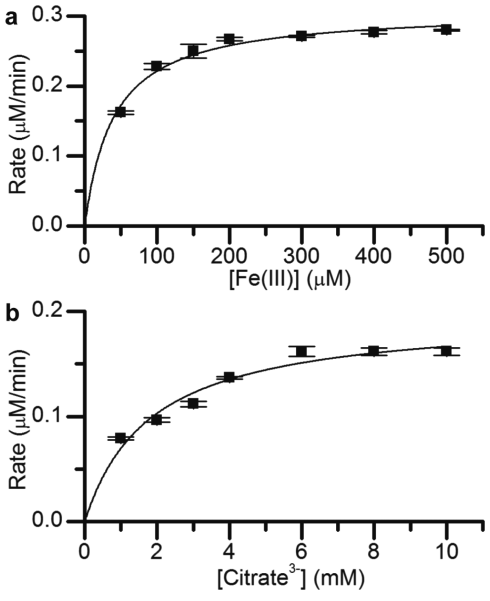

Figure 3. Initial rate dependence of $[FeHBED]^-$ formation on the concentrations of Fe(III) (a) and citrate^{3-} (b) from reaction of $[TiOHBED]^-$ and Fe(citrate)$_2^{5-}$. Hyperbolic growth dependence in the figures implicates the existence of an intermediate involving both Fe(III) and citrate^{3-}. Data were fit to eq 1 using nonlinear iterative regression.

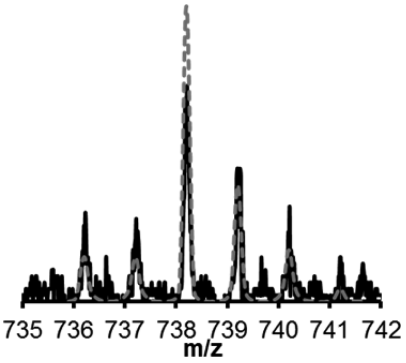

Figure 4. Negative-ion mass spectrum of the intermediate quaternary complex ($Na_2[TiO(HBED)Fe(Citrate)]^-$) detected in the kinetics study of $[FeHBED]^-$ formation. Theoretical isotope distribution (---) is overlaid on the experimental data (—).

Whether or not this spectrum provides a complete picture of the intermediate it suggests at the very least that the intermediate is composed of the four distinct components: Fe(III), Ti(IV), HBED, and citrate. This intermediate existed in the initial minutes of the multihour length of the reaction. Kinetics data were fitted to a mechanism where the quaternary complex intermediate (depicted with an unspecified charge state) forms before metal substitution occurs assuming steady state conditions for the intermediate (Scheme 1). The intermediate then dissociates, yielding $[FeHBED]^-$ followed by rapid formation of $[Ti(citrate)_3]^{8-}$. An alternative mechanism where citrate first removes Ti(IV) from HBED followed by HBED chelation of Fe(III) was ruled out based on a separate kinetics study of the reaction between metal-free HBED and $[Fe(citrate)_2]^{5-}$ at different citrate concentrations. This study showed that $[FeHBED]^-$ formation in the absence of Ti must occur via a different mechanism because the formation rates were significantly faster (3−24 times faster) and the rate dependence on citrate concentration showed an opposite effect. Rather than a hyperbolic growth dependence, the rates decreased as citrate

Scheme 1. Proposed Mechanism for Displacing Ti(IV) from HBED Coordination by Fe(III)

$$[TiOHBED]^- + [Fe(Citrate)_2]^{5-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [TiO(HBED)Fe(Citrate)]^a + Citrate^{3-}$$

$$[TiO(HBED)Fe(Citrate)]^a \xrightarrow{k_2} [FeHBED]^- + [TiO(Citrate)]^{2-}$$

$$[TiO(Citrate)]^{2-} + 2\ Citrate^{3-} \xrightarrow{k_3} [Ti(Citrate)_3]^{8-} + H2O$$

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concentration increased and plateaued at the upper concentration level (Figure S1, Supporting Information).

To derive a rate equation for Scheme 1, the equilibrium step was simplified by treating Fe(III) as independent from citrate when it interacts with $[TiOHBED]^-$ even though it exists in solution as a labile Fe(III) citrate complex.

$$[TiOHBED]^- + Fe(III) + Citrate^{3-} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [TiO(HBED)Fe(Citrate)]$$

It would be difficult to determine whether the citrate that attaches to Ti(IV) in the intermediate is originally Fe(III) free or Fe(III) bound. Mathematically, this generic representation of Fe(III) does not change the total number of Fe(III) and citrate expressed in the equilibrium step. The rate equation (eq 1) is obtained where k_2 is $(6.2 \pm 0.2) \times 10^{-3}$ min^{-1} and K is $(4.0 \pm 0.5) \times 10^5$ μM^2. For complete derivation of this equation, please refer to the Supporting Information.

$$\frac{d\{[FeHBED]^-\}}{dt} = \frac{k_2\{[TiOHBED]^-\}_{init}[Fe(III)][Citrate^{3-}]}{\frac{k_{-1}+k_2}{k_1} + [Fe(III)][Citrate^{3-}]} = \frac{k_2\{[TiOHBED]^-\}_{init}[Fe(III)][Citrate^{3-}]}{K + [Fe(III)][Citrate^{3-}]} \quad (1)$$

In total, displacement of Ti(IV) from HBED coordination by Fe(III) is a kinetically slow process taking place on the time scale of hours in the micromolar concentration range examined. This is a favorable finding because it is undesirable for metal displacement to occur so rapidly that $[TiOHBED]^-$ cannot survive transport through the bloodstream (assuming intravenous delivery) and into cells due to the presence of Fe(III) biomolecules. Importantly though, the reaction was fast enough relative to the time frame of the cytotoxicity experiments (3 days) that it is physiologically feasible for Ti(IV) release to occur within cells.

Cell Viability Studies Reveal the Importance of Fe(III) Depletion in the Proposed Drug Design Strategy and the Specificity of the Strategy for Ti(IV). The metal displacement kinetics study provided a physiologically relevant time scale for Ti(IV) transport by HBED and release inside cells, but it did not inform on whether HBED will play a detrimental iron depletion role. To examine this aspect of the proposed drug design strategy, the cytotoxic potencies of $HBED^-$, $[TiOHBED]^-$, $[FeHBED]^-$, and $[GaHBED]^-$ against A549 and MRC5 cells were measured at pH 7.4. Screened up to 100 μM concentration, $[FeHBED]^-$ and $[GaHBED]^-$ demonstrated insignificant activity against both cell lines whereas HBED displayed moderate activity against A549 cells ($IC_{50} = 105.2 \pm 13$ μM) and no activity against MRC5 cells (Table 1; Figure 5). $[TiOHBED]^-$ was most active against both cell lines. It was a superior cytotoxic agent against A549 lung cancer cells ($IC_{50} = 24.1 \pm 1.2$ μM) than against MRC5 lung normal cells ($IC_{50} = 42.0 \pm 4.4$ μM). These results strongly support the contention that HBED contributes to cytotoxicity by depleting cells of Fe(III). The greater antiproliferative effect that $[TiOHBED]^-$ and $HBED^-$ exhibit against the A549 cancer cells can be attributed to the higher requirement of cancer cells for iron.[20]

In support of the iron depletion hypothesis is the lack of cytotoxic behavior by $[FeHBED]^-$ because it is already Fe(III) saturated and unable to have any effect on Fe(III) levels in cells. It is therefore unsurprising that $[FeHBED]^-$ cannot induce cell death, whereas $[TiOHBED]^-$ and $HBED^-$, with their Fe(III) binding capacities, can. Further support for this hypothesis is provided by an Fe(III) rescue experiment. A549 and MRC5 cells were pretreated with 100 μM $[Fe(citrate)_2]^{5-}$ to determine whether addition of Fe(III) would help the cells resist the cytotoxic effects of equimolar amounts of $[TiOHBED]^-$ and $HBED^-$ (Figure 6). The added Fe(III) completely ablates the cytotoxicity displayed by $HBED^-$ against A549 cells, increasing the cell viability from $45.2 \pm 2.9\%$ to $93.0 \pm 8.7\%$ ($p < 0.01$). No difference in activity was observed against MRC5 as expected because $HBED^-$ does not induce cell death at the concentration examined. The supplemented Fe(III) attenuates the cytotoxicity of $[TiOHBED]^-$, increasing A549 cell viability from $8.0 \pm 0.4\%$ to $31.9 \pm 1.9\%$ ($p < 0.01$) and increasing MRC5 cell viability from $13.3 \pm 1.3\%$ to $41.6 \pm 4.7\%$ ($p < 0.01$). In the $[TiOHBED]^-$ case, full cell viability recovery is not observed because the additional Fe(III) cannot counteract the effect of the Ti(IV) ion.

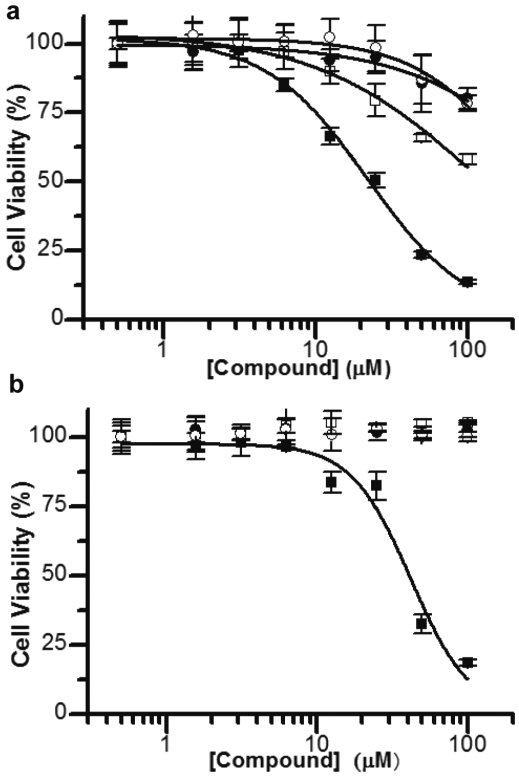

Figure 5. Dose–response curves for $[TiOHBED]^-$ (■), $HBED^-$ (□), $[FeHBED]^-$ (●), and $[GaHBED]^-$ (○) against A549 cancer (a) and MRC5 (b) normal human lung cells.

In addition to revealing that Fe(III) depletion is integral to the proposed drug design strategy, cell viability studies also elucidate specificity of the drug template for Ti(IV) activity. This is best illustrated with the results for $[GaHBED]^-$. Ga(III) has previously been shown to be cytotoxic,[31,39,40] even against A549 cells.[41] There has been great interest in developing Ga(III)-based anticancer drugs because of the ability of Ga(III) to biomimic Fe(III) and possibly effect Fe(III) biochemistry in a cellular detrimental fashion. Our work shows that Ga(III) formulation is critical to its cytotoxic behavior. $[GaHBED]^-$ is very similar to $[FeHBED]^-$ structurally and in terms of stability constants ($\log K_{FeHBED} = 39.01$ and $\log K_{FeHBED} = 38.51$).[27] It would have a poor Fe(III) binding capacity or rather a poor

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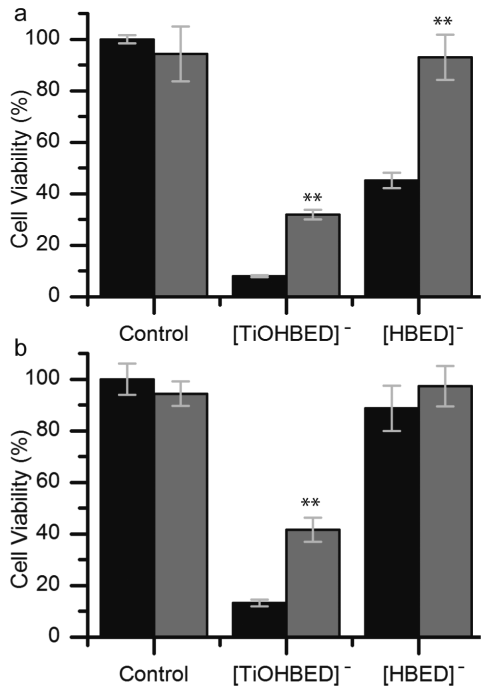

Figure 6. Result of the Fe(III) rescue experiment. A549 (a) and MRC5 (b) cell viabilities measured in the absence (black) and presence (gray) of 100 μM Fe(III) citrate. Added Fe(III) citrate improves A549 cell viability against treatment with equimolar amounts of $[TiOHBED]^-$ and $HBED^-$ (Student's *t* test, **, *p* value $\ll 0.01$, $N = 4$). Fe(III) citrate also improves MRC5 cell viability against treatment with equimolar $[TiOHBED]^-$ (Student's *t* test, **, *p* value $\ll 0.01$, $N = 4$).

ability to exchange Ga(III) with Fe(III) and should not affect cellular iron levels much at all. This concurs with the finding by Lundberg and Chitambar that Ga(III) in a nitrate form could kill HL60 cells but as a compound of HBED could not.[39] Interestingly, a synergistic cytotoxic effect was observed when first treating the cells with HBED and then with gallium nitrate. It is likely that HBED first depletes the cells of iron, and then when gallium nitrate is delivered Ga(III) binds to the Fe(III)-vacated sites resulting in adverse alteration of cellular metabolism. This drug delivery approach, though inefficient, highlights the potential of our drug design strategy. HBED, as a cTfm ligand, presents the appropriate coordination chemistry balance between stability and lability to effectively transport Ti(IV) into cells and to release Ti(IV) upon interaction with Fe(III) biomolecules (Figure 2).

This balance of stability and lability explains why any Fe(III) chelator would not work for our drug design strategy. In a previous A549 cell viability study, the Ti(IV) compounds of Fe(III) chelators citrate and naphthalene-2,3-diolate did not demonstrate any cytotoxic behavior.[13] The Ti(IV) citrate compound $[Ti(citrate)_3]^{8-}$ is highly hydrolysis prone and labile.[15,37] At serum citrate concentrations, $[Ti(citrate)_3]^{8-}$ will not survive as an intact compound and will likely lose Ti(IV) to binding by other molecules such as transferrin in the bloodstream before it would enter cells. Furthermore, citrate will serve as an ineffective chelator of Fe(III) for removal from cells. The Ti(IV) naphthalene-2,3-diolate compound $[Ti(naphthalene\text{-}2,3\text{-}diolate)_3]^{2-}$ will be expected to enter into cells, considering catechol-type ligands are generally effective transporting agents.[42] The ligand has very high affinity for Ti(IV),[16] likely exceeding its affinity for Fe(III) at pH 7.4 as it can coordinatively saturate Ti(IV) but not Fe(III) at physiological pH.[43] This strongly casts doubt as to the ability of naphthalene-2,3-diolate to release Ti(IV) in cells to bind and deplete Fe(III).

Our work with HBED as a case study for use of chemical Tf mimetics in development of a new anticancer Ti(IV)-based drug design strategy shows excellent promise. The compound $[TiOHBED]^-$ efficiently combines the synergistic dual function of disrupting cellular use of Fe(III) and facilitating Ti(IV) delivery to intracellular targets for cytotoxic effect. The next step is to explore the therapeutic application of other cTfm ligands. These ligands like the easily derivatized HBED ligand[44] can be structurally manipulated to enable selective targeting of cancer cells by the Ti(IV) compounds.

■ ASSOCIATED CONTENT

Supporting Information

Kinetics data for the rate dependence of [FeHBED]-formation on citrate concentration from the reaction of metal-free HBED and Fe(III) citrate is included. Also the derivation of eq 1 is shown. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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