Supporting Information

An iron chelator transmetalative approach to inhibit human ribonucleotide reductase

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1. Methods

1.1. EPR analysis of Fe(III) compounds. In situ 1 mM solutions of [Fe(Def)$_2$]$^{3-}$, [Fe(HBED)]$^-$, [Fe(Cit)(Def)]$^{4+}$, and [Fe(Cit)$_2$]$^{5-}$ were prepared in 50 mM HEPES buffer at pH 7.4 with 2% DMF (v/v). The samples were then transferred to EPR tubes and froze in liquid N$_2$ before EPR analysis. The EPR experiments were performed using the following conditions: microwave frequency, 9.338 GHz; microwave power, 2 mW; magnetic field modulation amplitude, 1 mT for high-spin Fe(III) (S = 5/2) detection, temperature: 20 K.

1.2. Cyclic voltammetry studies of Fe(III) compounds. In situ 5 mM aqueous solutions [Fe(Def)$_2$]$^{3-}$, [Fe(HBED)]$^-$, [Fe(Cit)(Def)]$^{4+}$, and [Fe(Cit)$_2$]$^{5-}$ were prepared at pH 7.4 having 0.1 M KCl and 2% DMF (v/v). A solution of Ti(Def)$_2$ was also prepared under these conditions. The samples were purged with N$_2$ for 15 min then analyzed under an N$_2$ stream using a potentiostat with the following conditions- number of scans: 4, scan rate: 100 mV/s Counter electrode: Pt; Working electrode: Au; Reference electrode: Ag/AgCl 3 M KCl (BASi, IN). Before the scanning, the Au working electrode surface was polished using alumina slurries from higher to lower sized particles on soft surface pads. After polishing, the electrode was rinsed well with the distilled water and sonicated for 10 min using ethanol/distilled water solution (50% v/v) to remove residual abrasive particles. A control of 0.1 M KCl(aq) with 2% DMF (v/v) at pH 7.4 was run before analyzing the samples to ensure the electrode was properly primed. The cyclic voltammograms were plotted following the IUPAC convention.

1.3. Determination of the formation constant of [Ti(Deferasirox)$_2$]$^{2-}$ ([Ti(Def)$_2$]$^{2-}$). A spectrophotometric ligand competition experiment was performed at pH 7.4, 25 °C, and I = 0.1 M (0.1 M KCl). All of the solutions were prepared in 100 mM HEPES buffer, which included 7 mM Nascitrate and 5% DMF (v/v). To separate 1 mL solutions of 26.7 μM [Ti(Citrate)$_3$]$^{8-}$ ([Ti(Cit)$_3$]$^8-$) were added varying concentrations of Deferasirox (Def$^-$) (0 to 417 μM). The solutions were equilibrated for 24 h and their absorbances were scanned using a UV-Vis spectrophotometer in the wavelength range of 300 to 600 nm. The formation constant for [Ti(Def)$_2$]$^{2-}$ was determined after accounting for all equilibria in solution as follows:

\[
K_{\text{Ti(Cit)}_3^{8-} + 2 \text{H}_2\text{Def}^{1-} \rightleftharpoons \text{Ti(Def)}_2^{2-} + 3 \text{HCit}^{3-} + \text{H}^+} = \frac{[\text{Ti(Def)}_2^{2-}][\text{HCit}^{3-}]^3[\text{H}^+]}{[\text{Ti(Cit)}_3^{8-}][\text{H}_2\text{Def}^{1-}]} \quad (eq. 1)
\]

\[
K = K_{\text{app}}[\text{HCit}^{3-}][\text{H}^+] \quad (eq. 2)
\]

where $K_{\text{app}}$ is an apparent binding constant and is represented by eq. 3.

\[
K_{\text{app}} = \frac{[\text{Ti(Def)}_2^{2-}]}{[\text{Ti(Cit)}_3^{8-}][\text{H}_2\text{Def}^{1-}]} \quad (eq. 3)
\]

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The total Ti(IV) concentration is defined by \([\text{Ti(Cit)}_3^{8-}]_{\text{initial}}\) and it is represented by the mass balance of all Ti(IV) species in equilibrium (eq. 4).

\[
[\text{Ti(Cit)}_3^{8-}]_{\text{int}} = [\text{Ti(Cit)}_3^{8-}]_{\text{eq}} + [\text{Ti(Def)}_2^{2-}]_{\text{eq}} \quad (\text{eq. 4})
\]

Eq. 4 is rearranged in terms of \([\text{Ti(Def)}_2^{2-}]\) to yield eq. 5.

\[
[\text{Ti(Def)}_2^{2-}]_{\text{eq}} = [\text{Ti(Cit)}_3^{8-}]_{\text{int}} - [\text{Ti(Cit)}_3^{8-}]_{\text{eq}} \quad (\text{eq. 5})
\]

The total mass balance of H\text{2Def}^{1-} is represented by eq. 6.

\[
[H_2\text{Def}^{1-}]_{\text{int}} = [H_2\text{Def}^{1-}]_{\text{eq}} + 2[\text{Ti(Def)}_2^{2-}]_{\text{eq}} \quad (\text{eq. 6})
\]

Eq. 6 is rearranged in terms of \([H_2\text{Def}^{1-}]_{\text{eq}}\) to give eq. 7.

\[
[H_2\text{Def}^{1-}]_{\text{eq}} = [H_2\text{Def}^{1-}]_{\text{int}} - 2[\text{Ti(Def)}_2^{2-}]_{\text{eq}} \quad (\text{eq. 7})
\]

Eq. 5 is substituted into eq. 3 to obtain eq. 8.

\[
\frac{1}{K_{\text{app}}[H_2\text{Def}^{1-}]_{\text{eq}}^2 + 1} = \frac{[\text{Ti(Cit)}_3^{8-}]_{\text{eq}}}{[\text{Ti(Cit)}_3^{8-}]_{\text{int}}} \quad (\text{eq. 8})
\]

Eq. 7 is substituted into eq. 8 to obtain eq. 9.

\[
\frac{1}{K_{\text{app}}([H_2\text{Def}^{1-}]_{\text{int}} - 2[\text{Ti(Def)}_2^{2-}]_{\text{eq}})^2 + 1} = \frac{[\text{Ti(Cit)}_3^{8-}]_{\text{eq}}}{[\text{Ti(Cit)}_3^{8-}]_{\text{int}}} \quad (\text{eq. 9})
\]

Finally eq. 10 is obtained by substituting eq. 5 into eq. 9.

\[
\frac{[\text{Ti(Cit)}_3^{8-}]_{\text{eq}}}{[\text{Ti(Cit)}_3^{8-}]_{\text{int}}} = \frac{1}{K_{\text{app}}([H_2\text{Def}^{1-}]_{\text{int}} - 2([\text{Ti(Cit)}_3^{8-}]_{\text{int}} - [\text{Ti(Cit)}_3^{8-}]_{\text{eq}})^2 + 1} \quad (\text{eq. 10})
\]

Eq. 10 can be simplified to eq. 11.

\[
y = \frac{1}{K_{\text{app}}x^2 + 1} \quad (\text{eq. 11})
\]

where \(x = [H_2\text{Def}^{1-}]_{\text{eq}}\) and \(y = \frac{[\text{Ti(Cit)}_3^{8-}]_{\text{eq}}}{[\text{Ti(Cit)}_3^{8-}]_{\text{int}}}\)

The value of \(K_{\text{app}}\) was calculated by determining all equilibria concentrations using the concentration of \([\text{Ti(Def)}_2^{2-}]_{\text{eq}}\) derived from the absorbance at 365 nm. Eq. 11 was iteratively fitted by nonlinear analysis using OriginPro8.5. The \(K_{\text{app}}\) value was determined to be \((62.9 \pm 9) \times 10^{-4}\) \(\mu\text{M}^2\). The value of \(K\) was then determined using the parameters of 7 mM citrate and pH 7.4.
\[ K = ((62.9 \pm 9) \times 10^9 \text{M}^{-2}) \times (0.007 \text{M})^3 \times (10^{-7.4} \text{M}) = (8.59 \pm 1.2) \times 10^{-5} \text{M}^2 \]

\[ \log K = -4.066 \pm 0.061 \]

| Equilibria                                                                 | \( \log K \) | Ref. |
|---------------------------------------------------------------------------|-------------|------|
| \( \text{Ti(Cit)}_3^{8-} + 2 \text{H}_2\text{Def}^{2-} \rightleftharpoons \text{Ti(Def)}_2^{2-} + 3 \text{HCit}^{3-} + \text{H}^+ \) | -4.066      |      |
| \( \text{Ti(IV)} + 3 \text{HCit}^{3-} \rightleftharpoons \text{Ti(Cit)}_3^{8-} + 3 \text{H}^+ \)             | 4.07        | 1    |
| 2 \text{Def}^{3-} + 4 \text{H}^+ \rightleftharpoons 2 \text{H}_2\text{Def}^{4-}                                             | 19.41       | 2    |
| \( \text{Ti(IV)} + 2 \text{Def}^{3-} \rightleftharpoons \text{Ti(Def)}_2^{2-} \)                                       | 38.8 ± 0.1  |      |

1.4. **Partition coefficient (log \( D_{7.4} \)) measurements of \([\text{Ti(Def)}_2]^{2-}\) and \([\text{TiO(H}^+\text{-HBED})]^{+}\).**

Partition coefficient (log \( D_{7.4} \)) measurements were performed adapting a literature protocol for the shake-flask procedure. Stock solutions of 2 mM neutral Ti(Def)_2 and 2 mM neutral Ti(HBED) were prepared in DMF. Solutions of 5, 10, 15, 20 and 25 \( \mu \text{M} \) of the Ti(IV) cTfm compounds in octanol with 5\% DMF (v/v) and 1X PBS (pH 7.4) with 5\% DMF (v/v) were prepared and scanned with a UV-Vis spectrophotometer. The absorbance maximum was plotted versus the corresponding concentrations to produce calibration curves. The calibration curves for \([\text{Ti(Def)}_2]^{2-}\) and \([\text{TiO(H}^+\text{-HBED})]^{+}\) were obtained at 372 nm and 373 nm in octanol with extinction coefficients of 13,500 M\(^{-1}\)cm\(^{-1}\) and 10,700 M\(^{-1}\)cm\(^{-1}\), respectively. The extinction coefficients in aqueous solution at pH 7.4 were previously reported although the values were experimentally determined in this work to account for any background differences. Solutions of 25 \( \mu \text{M} \) of the Ti(IV) compounds with 5\% DMF (v/v) in equal volume of 1X PBS at pH 7.4 and octanol were prepared and left shaking overnight to equilibrate. The octanol layer was carefully extracted, and its absorbance was measured. Using the calibration curves, the concentration of the Ti(IV) compounds in the octanol and aqueous layers were obtained. The log\( D_{7.4} \) was calculated by obtaining the logarithm of the concentration of the compound in octanol divided by the concentration in the aqueous layer using the equation:

\[ \log D_{7.4} = \log([\text{Compound}_{\text{octanol}}]/[\text{Compound}_{\text{aqueous\ layer}}]) \]

The log \( D_{7.4} \) measurements for each Ti(IV) compound were done in triplicate.

1.5. **Hemolysis assay to evaluate the interaction of \([\text{Ti(Def)}_2]^{2-}\) and \([\text{TiO(H}^+\text{-HBED})]^{+}\) with erythrocytes.** Single donor human red blood cells were centrifuged at 2000 rpm for 5 min and levels of hematocrit (red, lower layer) and Alsever’s solution (upper layer) were marked on the tube. The supernatant was gently aspirated and added into bleach to later be discarded into biohazardous waste. Cells were washed with 150 mM NaCl and centrifuged as described twice. After a final step of centrifugation, 1X PBS at pH 7.4 was added to the cells until the supernatant
mark and then the cells were resuspended. 100 μL of a 1:25 dilution of the erythrocytes was added to V-bottom 96-well plate. Stocks solutions of [Ti(Def)]2+ (20, 100, and 200 μM), Def+ (40, 200, and 400 μM), [TiO(H−-HBED)]+ (20, 100 and 200 μM) and HBED− (20, 100, and 200 μM) were prepared in 1X PBS at pH 7.4 containing 2% DMF (v/v). 100 μL of each stock solution was added to the red blood cell containing wells. For the positive control, 100 μL of 10% Triton X-100 containing 2% DMF (v/v) was added. For the negative control, 100 μL of 1X PBS (pH 7.4, 2% DMF(v/v)) was added. All of the compound treatments and negative and positive controls were done at N=4. The plate was incubated at 37 ºC for 1 h and centrifuged for 5 min at 2000 rpm to pellet intact erythrocytes. The supernatant of all of the wells was transferred into a clear, flat-bottomed 96-well plate and the absorbance at 420 nm was measured in the Tecan Infinite M200 PRO plate reader. The results are expressed as the percentage of hemolysis, calculated using the equation:

\[ \% \text{ Hemolysis} = 100 \times \frac{(\text{ABS of sample})-(\text{ABS of neg control})}{(\text{ABS of pos control})-(\text{ABS of neg control})} \]

1.6. Transmetalation reaction of [Ti(Def)]2+ and [TiO(H−-HBED)]+ with methemoglobin. To 12.5 μM Fe(III)-saturated hemoglobin (methemoglobin, MHB) was reacted 50 μM [Ti(Def)]2+, 50 μM [TiO(H−-HBED)], 50 μM HBED− and 100 μM Def+ (1X PBS buffer, 1% DMF (v/v), pH 7.4). Every 24 h during a period of 96 h, the solutions were scanned by UV-Vis in the 350 to 700 nm wavelength range.

1.7. Synthesis and characterization of Ti6O7(C10H14N5O12P3)5(H2O)34 (Ti6O7(ATP)5(H2O)34). An aqueous solution of ATP was prepared at the concentration of 120 mM. The solution was cooled in an ice bath purged with argon gas for 15 min while stirring. To this solution TiCl4 was added dropwise to achieve a molar ratio of 1:6; Ti:ATP. A white precipitate immediately formed with >90% yield. The reaction mixture was then left to equilibrate for an additional h opened to the air. The final pH of the solution was 1.34. After 1 h, the suspension was transferred to a falcon tube and centrifuged at 11,000 rpm for 10 min. The pellet obtained was washed with autoclaved water through three centrifuge cycles. The product after purification gave ~90% yield. The pellet was dried overnight using speedvac. Quantitative C, H, and N elemental analysis was performed by Atlantic Microlabs (Norcross, GA). The % theoretical/ found for the product obtained was % C 16.60/16.45; % H 3.84/3.57; % N 9.68/9.31. Titanium quantification (% theoretical/ found; % Ti 7.94/7.82) was performed by a Ti(IV) colorimetric assay. These elemental analyses fit the molecular formulation of Ti6O7(C10H14N5O12P3)5(H2O)34 with a molecular weight of 3,617.55 g/mol. The qualitative elemental composition was determined by EDS-SEM, which confirmed the presence of the C, H, N, O, P, and Ti elements. The SEM image and ED spectrum were obtained using SEM at an accelerating voltage of 20 kV and a magnification of 15000 X. The analysis was performed using 1 mg of the powdered sample which was evenly distributed on an ultrathin carbon film and coated with gold. The solid-state product obtained was then characterized by FTIR spectroscopy. Sample analysis was done in the KBr pellet form. Homogenized ground powders (sample and KBr) were pressed to make the pellets using a SpectraTech Macro-Micro KBr Die Kit and a Carver 12 tons hydraulic press (Wabash, IN). The pellets were then kept in a desiccator for 2 h before analysis. A total of 256 acquisitions were performed at a resolution of 4 cm−1 over the range 4000-500 cm−1. FTIR studies demonstrated the characteristic band shifting due to metal phosphate binding in the Ti-ATP complex. In the spectra of pure ATP, the region from 1270-900
970 cm\(^{-1}\) is associated with different types of vibrations in phosphate groups.\(^{6-7}\) The vibrational bands of the pyrophosphate (P-O-P) linkage between \(\alpha\), \(\beta\), and \(\gamma\) phosphate groups are represented by two maxima at 914 and 899 cm\(^{-1}\). The sharp bands at 1105-1125 cm\(^{-1}\) are associated with the symmetric O-P-O vibrations in \(\alpha\) and \(\beta\) phosphate groups together with the anti-symmetric vibrations of the same groups at 1222-1257 cm\(^{-1}\). There is a low-intensity small band at 1163 cm\(^{-1}\) which represents the asymmetric vibrations of O-P-O in \(\gamma\) phosphate group. The peaks at 997 cm\(^{-1}\) and 955 cm\(^{-1}\) are the antisymmetric and symmetric P-O vibrations of the terminal phosphate group of the pure ATP. The sharp small peaks at 997, 1018, and 1045 cm\(^{-1}\) are linked to the symmetric and anti-symmetric P-O and C-O vibrations. The broad band at 1090 cm\(^{-1}\), a sharp peak at 970 cm\(^{-1}\) and shoulders at 950 and 875 cm\(^{-1}\) are the vibration of protonated phosphate (P-O-H) groups. These vibrational bands for the protonated phosphate groups are absent in the Ti-ATP complex indicating the coordination through these phosphate groups of the ATP. However, due to the substantial overlapping of different other vibrations, it is difficult to specify, whether the coordination is bi or tridentate. Additionally, the band associated with the asymmetric vibrations of O-P-O in \(\gamma\) at 1163 cm\(^{-1}\) is not present indicating the involvement of \(\gamma\) phosphate in the coordination. The peaks corresponding to the antisymmetric vibrations of O-P-O at 1222 and 1257 cm\(^{-1}\) in \(\alpha\) and \(\beta\) phosphate groups are broadened and shifted (one peak by \(~3\) cm\(^{-1}\)) indicating the participation of either \(\alpha\) and/or \(\beta\) phosphate groups in the coordination by Ti(IV). Powder X-Ray diffraction was also performed using the powdered solid. Powder X-ray diffractograms were collected at 300K using a Rigaku XtalLAB SuperNova single micro-focus Cu-K\(\alpha\) radiation (\(\lambda=1.5417\) Å) source equipped with a HyPix3000 X-ray detector in transmission mode operating at 50 kV and 1 mA. Powdered sample was mounted in MiTeGen micro loops. Powder diffractograms were collected over an angular 2-theta (\(\theta\)) range of 5–50\(^{\circ}\) with a step of 0.01\(^{\circ}\) using Gandolfi move for powder. Data was analyzed within the CrystallisPro software ver. 1.171.39.43c.\(^{8}\) The solution state characterization of the compound was performed by \(^{31}\)P NMR spectroscopy. The sample was prepared by dissolving in 1 mL of 27 mM sodium bicarbonate buffer (5 mM in terms of ATP concentration) at pH 7.4 (using 10\% D\(_2\)O (v/v)). A Ti(IV) free ATP sample (5 mM) was prepared in an identical manner. \(^{31}\)P-NMR recordings were carried out on a Bruker Ascend Aeon 700 NMR spectrometer with a 10 mm direct Z123952_0041 (CP QCI 700S3 H-P/C/N-D-05 Z) probe at 298.1K. The spectra were acquired after 1 h and 24 h (to achieve equilibration) in solution using the following parameters: NS = 678, SWH 11.4 kHz, Relaxation delay 2s, Pulse = 1.5 \(\mu\)sec, Phase 41.6 degree. The spectra were collected in an Wilmad Insert tube referenced internally with 5 mM H\(_3\)PO\(_4\) (prepared in 10\% D\(_2\)O v/v) by setting the SR frequency to 0 ppm. Spectra were acquired and processed using the Bruker Topsis 3.6 software. Other \(^{31}\)P NMR experiments were also performed in this same manner using the internal standard of 5 mM H\(_3\)PO\(_4\).

1.8. The reaction of Ti(Cit)\(^{3+}\) and nucleotides AMP, ADP, and ATP monitored by \(^{31}\)P NMR studies. 1 mM [Ti(Cit)\(^{3+}\)] was separately reacted with 5 mM ATP, 5 mM ADP, and 5 mM AMP in 27 mM sodium bicarbonate buffer at pH 7.4 (using 10\% D\(_2\)O (v/v)) for 24 h. After 1 h and 24 h, \(^{31}\)P-NMR spectra were collected of the 5 mM stock solutions of ATP (as already noted above), ADP, and AMP and of the reaction solution using parameters described above.

1.9. DNA interaction studies with [Ti(Def)\(^{2+}\)] and [TiO(H\(^{+}\)-HBED)]\(^{-}\). 2\(\times\)10\(^{6}\) cells/mL Jurkat cells (5 mL) were treated with 50 \(\mu\)L compounds for 3, 10 and 24 h with a final concentration of 19 \(\mu\)M of [Ti(Def)\(^{2+}\)], 29 \(\mu\)M Def, 27 \(\mu\)M HBED\(^{2-}\) and 27 \(\mu\)M [TiO(H\(^{+}\)-HBED)]\(^{-}\) (0.5\% DMF (v/v)) in 1X PBS. After incubation, the cells were washed twice with 1X PBS buffer and
centrifuged at 1200 rpm. Cell pellets were resuspended in 1 mL of DNAzol Reagent. The samples were agitated and the lysate was transferred into a 2 mL centrifuge tube. The homogenate was centrifuged at 10,000 rpm for 10 min at 25 °C and the supernatant was used for precipitating the DNA. 1 mL of DNAzol and ethanol mix (0.5 mL of 100% ethanol per 1 DNAzol) was added to each sample. The solution was mixed by inverting the tube 8-10 times and stored at room temperature for 3 min. DNA should quickly become visible as a cloudy precipitate. The DNA precipitate was collected with a pipette tip and transferred it to a clean tube. The precipitate was washed three times with 0.9 mL of 75% ethanol (v/v). At each wash, the DNA was suspended in ethanol by inverting the tubes 3-6 times. The tubes were kept vertically for 0.5-1 min to allow the DNA to settle to the bottom of the tubes and the ethanol was removed. The DNA was air dried by storing in an open tube for 5-15 seconds after removing the ethanol. The DNA was dissolved in 200 µL of 8 mM NaOH by slowly passing the pellet through a pipette tip. 20.2 µL of 0.1M HEPES (free acid) was added to the samples to obtain the desired pH 8. After ensuring the purity of DNA by nanodrop UV Vis analysis, the samples were loaded onto a 1.5% agarose gel containing 1:10,000 part of GelRed nucleic acid stain. The gel was run at 80 V for 1-1.5 h and photographed under UV illumination. To investigate the possibility of Ti-DNA adduct formation, all of the DNA samples were analyzed using inductively coupled plasma - optical emission spectrometry (ICP-OES). Briefly, a linear calibration curve of Ti atomic standard solutions (in 2% HNO₃ (v/v)), at the concentration range from 1 to 100 ppb vs. intensity was prepared. 2% HNO₃ (v/v) was used as a blank. All of the DNA samples were diluted in 2% HNO₃ (v/v) to the final concentration of 50 ppb or 0.05 ng/µL. From the calibration curve, the amount of Ti present in the DNA samples was calculated.

1.10. NCI-60 cell one-dose screen. Ti(HBED) and Ti(Def)₂ (in neutral form) were sent to the National Cancer Institute for human tumor cell lines screening. The screening is done using the standard protocols at pH 7 and the Ti(IV) compounds are expected to speciate accordingly.⁹ The analysis allows the detection of both growth inhibition and lethality of the compounds. The official website can be visited for any additional information. (https://dtp.cancer.gov/discovery_development/nci-60/default.htm).

1.11. Jurkat cell culturing. Jurkat cells are nonadherent cells and they were cultured in an RPMI-1640 media (supplemented with 10% FBS (v/v) and 1% antibiotic solution (v/v) of penicillin/streptomycin) in non tissue culture treated 25 mL flasks according to the known protocol provided by the supplier ATCC. The cell passaging was done at 70% confluency by taking 3 mL of cells and diluting to 25 mL of fresh media. The cells were incubated at 37 °C in 5% CO₂ atmosphere. Upon obtaining the cell line, fresh stocks were prepared treating them with the 1% antibiotic solution (v/v) of penicillin/streptomycin and the mycoplasma removal agent and then washed and stored in the cryogen-compatible solution in a quantity to fill at least half of a cryobox. The stocks were stored in a -200 °C liquid nitrogen storage container. Each stock used in this study was tested to validate growth of the cells by comparing their doubling time according to company specifications and by visually evaluating their morphology following every passage. Cells were passaged until they reached the passage number deemed by the company the limit for avoiding any mutations. Cells were discarded even before this limit if they began to grow slowly and exhibit significant morphology changes.
1.12. Cell viability studies. 50 μL of Jurkat cells with a cell count of 1×10^5 cells/mL were placed in different wells of a 96 well plate. The cells were treated with 50 μL of [Ti(Def)_2]^{2-}, Def, [TiO(H^+-HBED)]^+, HBED^{2-} and cisplatin for 24 h and/or 48 h. Stock solutions of the compounds were prepared in DMF and then diluted in 1X PBS buffer to yield the concentration range of 0.1 to 100 μM. DMF in all final solutions was maintained at 1% v/v. The cells were also co-treated with [Ti(Def)_2]^{2-} and cisplatin in a 1:1 and 1:2 mole equivalent ratio for 48 h over the same concentration range but as a combined concentration of both compounds. A control group of cells was treated with buffer alone which included 1% DMF (v/v). At 4 h before completion of the incubation time, 25 μL of MTT solution (sterile-filtered 5 mg/mL solution, dissolved in 1X PBS buffer) was added to each well. During addition of the MTT solution, the plates were protected from light. After completion, 75 μL of 4% (w/v) SDS solution (sterile-filtered, dissolved in Tris buffer 1M, pH 10) was added to each well and incubated for 12 h. The absorbance of each well was measured at 570 nm and 800 nm (background control) on an Infinite M200 PRO Tecan Microplate Reader. Nonlinear regression in Origin 8.5 was utilized to fit the growth curve using the pharmacology dose response equation over the various drug concentrations to determine the half-maximal inhibitory concentration (IC_{50}) and the corresponding standard deviation. For the co-treatment experiments, the IC_{50} values were determined based on the combined concentration range of the corresponding compounds and the standard deviation. This information was used to generate an isobologram plot.\textsuperscript{11-12} The isobologram is a 2D plot in which each axis represents the concentrations (μM) of one of the compounds, and the corresponding IC_{50} values are plotted as points on the axes (intercepts). Then a diagonal line, called an additive isobal, is drawn, which joins the intercepts. The additive isobal is accompanied by dashed lines that define the error range. The error range is plotted by joining the standard deviation values, also plotted as points on the axes. Finally, the results of the isobolographic analysis can be easily visualized: if the plotted relative IC_{50} is located below or above the isobals, then the activity will be described as synergistic or antagonistic, respectively. If the plotted relative IC_{50} value is located inside the error range of the additive isobal, the activity can be described as additive. The co-treatment data were also analyzed by calculating the IC_{50} combination index (CI). CI = D_1/D_{IC501} + D_2/D_{IC502}, where D_1 and D_2 are the IC_{50} values for the separate compounds and D_{IC501} and D_{IC502} are the concentrations of the two compounds that combined exert an IC_{50} effect. CI <1, =1, and >1 indicate synergism, additivity, and antagonism, respectively. All samples were tested four times.

1.13. Cell cycle analysis by flow cytometry. 2×10^5 Jurkat cells/mL in a volume of 2.950 mL media were treated with the 50 μL of 29 μM Def, 18.8 μM [Ti(Def)_2]^{2-}, 26.3 μM HBED^{2-} and [TiO(H^+-HBED)]^+ for 3, 10, and 24 h. After incubation, the cells were transferred from the culture plate to 15 mL conical tubes. The samples were then centrifuged (~3 mL) at 1200 rpm for 5 min at room temperature and the supernatant was discarded. The pellet was resuspended in 3 mL warm (37 °C) 1X PBS buffer by gently pipetting up and down a few times and centrifuged at 1200 rpm for 5 min. After discarding the supernatant, the pellet was resuspended in 2.5 mL warm 1X PBS buffer and incubated for 20 min at 37 °C in the dark by agitating it every 5 min. After incubation, centrifugation was performed at 1200 rpm for 5 min at room temperature. The supernatant was discarded and the residual supernatant was drained for a few seconds by placing the inverted tube directly on a paper towel. The cells were then fixed by adding 700 μL of ethanol (70% v/v) dropwise (10 μL at once) by constant vortexing. The samples were incubated on ice for 15 min. At this stage, the samples were stored at 4 °C for 24 h. The cells were then centrifuged at 1200 rpm for 5 min and washed with 500 μL 1X PBS and centrifuged again at 1200 rpm for 5 min. The
supernatant was then discarded and the cell pellet was resuspended in 200 μL propidium iodide (PI) solution containing 50 μg/mL PI, 0.1 mg/mL RNase A, and 0.05% Triton X-100 (v/v) (prepared in 1X PBS buffer). The cells were incubated for 30 min in the dark at 37 °C and agitated every 10 min. After incubation, the samples were analyzed using the flow cytometer to determine the effect of the compounds on the cell cycle.

1.14. Cell apoptosis studies. 737.5 μL cells at a density of 1×10^6 cells/mL were treated with 12.5 μL of 20 μM Cisplatin, 50 μM [Ti(Cit)3]8-, 19 μM [Ti(Def)2]2-, 27 μM [TiO(H+-HBED)]-, 29 μM Def-, 27 μM HBED- and 1X PBS control with 0.5% DMF (v/v) in a 24 well plate for 24 and 72 h. All of the samples were prepared in triplicate. After incubation, the cells were harvested and washed twice with cold 1X PBS at 1200 rpm for 5 min at 4 °C. The washed cells were then resuspended in annexin-binding buffer (500 μL). The cell density was determined and diluted to get the density to ~1×10^6 cells/mL. Transferred 100 μL of the solution (1×10^5 cells) to an Eppendorf tube and added 5 μL of Annexin V Alexa Fluor conjugate and 1 μL PI (100 μg/mL) then gently vortexed the solution and incubated at room temperature for 15 min in the dark. After the incubation, 400 μl of the annexin-binding buffer was mixed and the samples were kept on ice. The samples were then analyzed by the flow cytometer by measuring the fluorescence emission using filters FL1 (Annexin V Alexa Fluor conjugate) and FL3 (PI).

1.15. Statistical analysis. Statistical analysis was performed using two-way ANOVA and Dunnett’s multiple comparison test. The error bars indicate standard deviation. p-values ≤ 0.05 vs control group were considered statistically significant.
2. Supporting Figures and Tables.

Figure S1. pH dependent speciation of the Ti(IV) cTfm complexes. Speciation models for 50 μM Ti(IV) in interaction with (a) Deferasirox (1:2 metal:ligand ratio) and with (b) HBED (1:1 metal:ligand ratio).\textsuperscript{4,5,13}

Figure S2. Ligand competition assay to determine the [Ti(Def)\textsubscript{2}]\textsuperscript{2-} formation constant at pH 7.4.
Figure S3. Dose response curves. Curves showing the 24 h treatment of Jurkat cells in the presence of $[\text{Ti(Def)}_2]^{2-}$ and $[\text{TiO(H}^+\text{-HBED})]$ and their metal-free ligands.

Figure S4. EPR spectra. Spectra of the in situ prepared solutions of 1 mM $[\text{Fe(Cit)}_2]$²⁻, $[\text{Fe(Cit)(Def)}]^4$⁻, $[\text{Fe(Def)}_2]^3$⁻, and $[\text{Fe(HBED)}]^-$ at pH 7.4. Experimental conditions: microwave frequency, 9.338 GHz; microwave power, 2 mW; magnetic field modulation amplitude, 1mT for high-spin Fe(III) ($S = 5/2$), temperature: 20 K.
**Figure S5.** Negative ion electrospray MS spectrum. In situ prepared $[\text{Fe(Citrate)(Def)}]^4$- shows the presence of $[\text{Fe(Citrate)(Def)}]^4 + 3\text{H}^+ ([\text{Fe(C}_6\text{H}_4\text{O}_7](\text{C}_2\text{H}_12\text{N}_3\text{O}_4))^4 + 3\text{H}^+$, m/z 616.97) and $[\text{Fe(Def)}]^3 + 2\text{H}^+ ([\text{Fe(C}_2\text{H}_12\text{N}_3\text{O}_4]^3 + 2\text{H}^+ , \text{m/z 798.02})$. The experimental data (solid line) overlay the theoretical isotope distribution (dotted line).

**Figure S6.** CV analysis. Proposed transmetalation reactions between Ti(IV) cTfm compounds and $[\text{Fe(Cit)}]^5$ at pH 7.4 demonstrated in terms of reduction potential values ($E_{1/2}$) or $E_c$ determined by cyclic voltammetry.
Figure S7. Hemolysis assay to evaluate the interaction of [Ti(Def)₂]²⁻ and [TiO(H⁺-HBED)]⁻ with erythrocytes. Evaluation of the hemolytic activity of [Ti(Def)₂]²⁻ and [TiO(H⁺-HBED)]⁻ and their metal-free ligands. Positive control: Triton X-100.

Figure S8. Transmetalation reaction of [Ti(Def)₂]²⁻ and [TiO(H⁺-HBED)]⁻ with Mhb. UV-Vis absorption spectra of the transmetallation reaction of 50 μM [Ti(Def)₂]²⁻, 50 μM [TiO(H⁺-HBED)]⁻, 50 μM HBED²⁻, and 100 μM Def with 12.5 μM Mhb at pH 7.4.
**Figure S9.** Dose-response curves. Cell viability of Jurkat cells treated with [Ti(Def)$_2$]$^2^-$ and cisplatin separately and in the co-treatment groups in the 1:1 and 1:2 ([Ti(Def)$_2$]$^2^-$:cisplatin) molar ratios for 48 h. The concentrations reported for the co-treatment groups are combined concentrations of [Ti(Def)$_2$]$^2^-$ and cisplatin.
Figure S10. Titanium(IV) phosphoryl interactions. (a) Three types (i-iii) of Ti(IV) binding to the phospho backbone of DNA. Type i and ii correspond to the titanocenyl moiety. Type i has been proposed for titanocene dichloride at pH 5.3 from direct interactions between a DNA fragment and the compound\textsuperscript{14} and for titanocene Y, an aqueous solution stable compound.\textsuperscript{15} Type ii has been proposed for titanocene dichloride at pH 7.0 from direct interactions between a DNA fragment and the compound.\textsuperscript{14} Type iii is a possible Ti(IV) coordination modality due to the physiological ligand dissociation of the highly unstable titanocene dichloride. (b) Proposed nucleotide coordination of Ti(IV) at ligand:Ti (L:Ti) ratios ≥ 3 and ≤ 1 using adenosine as representative nucleoside moiety. At L:Ti ratios ≤ 1, Ti(IV)-induced hydrolysis of the phosphate group is possible.
Figure S11. DNA gel electrophoresis. DNA electrophoresis for the studies of DNA interaction with [TiO(H⁺-HBED)]⁺, [Ti(Def)₂]²⁻, and the metal free ligands using 1.5% agarose gel. DNA was isolated from Jurkat cells after treatment with buffer control (A), 29 µM Def (B), 19 µM [Ti(Def)₂]²⁻ (C), 27 µM HBED²⁻ (D), and 27 µM [TiO(H⁺-HBED)]⁺ (E) for 3, 10 and 24 h.

Figure S12. Characterization of the Ti₆O₇(C₁₀H₁₄N₅O₁₃P₃)₅(H₂O)₃₄ compound. Scanning electron microscopy image of Ti₆O₇(C₁₀H₁₄N₅O₁₃P₃)₅(H₂O)₃₄ showing distribution of amorphous particles (a) Energy-dispersive X-ray spectrogram peak patterns of the corresponding elements present in the compound (b) Powder X-ray diffraction diagram (c) of ATP (gray) and Ti₆O₇(C₁₀H₁₄N₅O₁₃P₃)₅(H₂O)₃₄ (black).
Figure S13. FTIR spectra. IR spectra of Ti₆O₇(ATP)₅(H₂O)₃₄ (in olive green color) and the disodium salt of ATP disodium salt (in black color).

Figure S14. Zoomed-in sections (a-c) of the $^{31}$P NMR spectra for Ti₆O₇(ATP)₅(H₂O)₃₄ (top) and ATP (bottom) at pH 7.4 and 5 mM concentration based on ATP content (see Figure 5).
Figure S15. $^{31}$P NMR spectra. The spectra of (a) $\text{Ti}_6\text{O}_7(\text{ATP})_5(\text{H}_2\text{O})_{34}$, (b) Ti(IV) with 5 mM ATP (1:5), (c) 5 mM ATP, (d) Ti(IV) with 5 mM ADP (1:5), (e) 5 mM ADP, (f) Ti(IV) with 5 mM AMP, and (g) 5 mM AMP.
| Property                      | Value                                      |
|-------------------------------|--------------------------------------------|
| **Empirical formula**         | C₄₂H₂₆N₆O₈Ti                               |
| **Formula weight**            | 790.59 g/mol                               |
| **Temperature**              | 100 K                                      |
| **Wavelength**               | 1.54184 Å                                  |
| **Crystal system, space group** | Triclinic, P-1                           |
| **Unit cell dimensions**     |                                           |
| a                            | 12.2524(2) Å                               |
| α                            | 111.308(2) °                               |
| b                            | 14.8895(3) Å                               |
| β                            | 104.390(2) °                               |
| c                            | 15.0625(3) Å                               |
| γ                            | 99.092(2) °                                |
| **Volume**                   | 2383.98(9) Å³                             |
| **Z, calculated density**    | 2, 1.101 g/cm³                             |
| **Absorption coefficient**   | 1.943 mm⁻¹                                 |
| **F(000)**                   | 812.0                                      |
| **Crystal size**             | 0.133 × 0.08 × 0.049 mm                     |
| **Theta range for data collection** | 6.628 to 137.756°                      |
| **Limiting indices**         | -14 ≤ h ≤ 12, -17 ≤ k ≤ 18, -17 ≤ l ≤ 18 |
| **Reflections collected/unique** | 48,025/8773 (Rint = 0.0354)            |
| **Completeness to theta = 137.756°** | 99.9%                                     |
| **Absortion**                | Gaussian                                   |
| **Max. And min. transmission** | 1.000 and 0.882                           |
| **Refinement method**        | Full-matrix least squares on F²           |
| **Data/restraints/parameters** | 8773/0/516                               |
| **Goodness-of-fit on F²**    | 1.098                                      |
| **Final R indices [I > 2sigma(I)]** | R₁ = 0.0547, wR₂ = 0.1847         |
| **R indices (all data)**     | R₁ = 0.0622, wR₂ = 0.1901                 |
| **Largest diff. peak and hole** | 0.29 and -0.43 e Å⁻³               |
Table S2. Selected bond lengths (Å) and angles (°) for neutral Ti(Def)$_2$ with standard uncertainties in parentheses.

| Bond Lengths | Value (Å) |
|--------------|-----------|
| Ti(1)—O(1A) | 1.880(2)  |
| Ti(1)—O(2)  | 1.875(2)  |
| Ti(1)—O(1)  | 1.886(2)  |
| Ti(1)—O2A   | 1.905(2)  |
| Ti(1)—N(1A) | 2.148(2)  |
| Ti(1)—N(1)  | 2.141(2)  |
| O(1A)—Ti(1)—O(1) | 90.80(10) |
| O(1A)—Ti(1)—O(2A) | 163.37(9) |
| O(1A)—Ti(1)—N(1A) | 82.14(9)  |
| O(1A)—Ti(1)—N(1) | 102.43(9) |
| O(2)—Ti(1)—O(1A) | 90.78(9)  |
| O(2)—Ti(1)—O(1)  | 163.16(9) |
| O(2)—Ti(1)—O(2A) | 92.32(10) |
| O(2)—Ti(1)—N(1A) | 103.09(9) |
| O(2)—Ti(1)—N(1)  | 81.57(9)  |
| O(1)—Ti(1)—O(2A) | 90.95(10) |
| O(1)—Ti(1)—N(1A) | 93.73(9)  |
| O(1)—Ti(1)—N(1)  | 81.72(9)  |
| O(2A)—Ti(1)—N(1A)| 81.25(9)  |
| O(2A)—Ti(1)—N(1) | 94.18(9)  |
| N(1)—Ti(1)—N(1A) | 173.54(9) |

Table S3. A summary of the NCI 60 cancer cell line viability screen against 10 μM of [TiO(H+·HBED)]$^-$ and [Ti(Def)$_2$]$^{2-}$ for 48 h. The data are presented as the mean per cell line type.

| Cancer Cell Line Type | % Mean Cell Growth |
|-----------------------|--------------------|
|                       | Ti(Def)$_2$ | Ti(HBED) |
| Leukemia              | 34.7       | 61.1     |
| Non-Small Cell Lung   | 66.2       | 89.9     |
| Colon                 | 51.5       | 81.2     |
| CNS                   | 43.6       | 84.2     |
| Melanoma              | 52.0       | 86.4     |
| Ovarian               | 62.7       | 81.8     |
| Renal                 | 67.2       | 83.7     |
| Prostate              | 65.2       | 86.3     |
| Breast                | 56.0       | 82.6     |
Table S4. The IC\textsubscript{50} values for the viability of Jurkat cells treated with [TiO(H\textsuperscript{+}-HBED)]\textsuperscript{−} (24 h), [Ti(Def)]\textsubscript{2}\textsuperscript{2−} (24 h and 48 h), the metal-free ligands (24 h), and cisplatin (24 h and 48 h) at pH 7.4. Co-treatment experiments for 48 h were performed with [Ti(Def)]\textsubscript{2}\textsuperscript{2−} and cisplatin.

| Compounds                          | Time duration (h) | IC\textsubscript{50} ± SE (µM) |
|-----------------------------------|-------------------|-------------------------------|
| [Ti(Def)]\textsubscript{2}\textsuperscript{2−} | 24                | 9.1 ± 1.5                     |
| [Ti(Def)]\textsubscript{2}\textsuperscript{2−} | 48                | 3.40 ± 0.76                   |
| Def                               | 24                | 46.8 ± 1.1                    |
| [TiO(H\textsuperscript{+}-HBED)]\textsuperscript{−} | 24                | 24.7 ± 1.2                    |
| HBED\textsuperscript{2−}          | 24                | 53.2 ± 1.1                    |
| Cisplatin                         | 24                | 16.6 ± 8.6                    |
| Cisplatin                         | 48                | 1.68 ± 0.15                   |
| Cisplatin/ [Ti(Def)]\textsubscript{2}\textsuperscript{2−} = 1 | 48                | 2.14 ± 0.23                   |
| Cisplatin/ [Ti(Def)]\textsubscript{2}\textsuperscript{2−} = 2 | 48                | 1.01 ± 0.22                   |
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