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Cooperative Stabilization of Zn²⁺:DNA Complexes Through Netropsin Binding in the Minor Groove of FdU-Substituted DNA

Supratim Ghosh^{1,2}, Freddie R. Salsbury Jr.³, David A. Horita⁴, and William H. Gmeiner^{1,2}

¹Department of Cancer Biology, Wake Forest University School of Medicine

²Program in Molecular Genetics, Wake Forest University School of Medicine

³Department of Physics, Wake Forest University, Winston-Salem, NC

⁴Department of Biochemistry, Wake Forest University School of Medicine

Abstract

The simultaneous binding of netropsin in the minor groove and Zn²⁺ in the major groove of a DNA hairpin that includes 10 consecutive FdU nucleotides at the 3'-terminus (3'FdU) was demonstrated based upon NMR spectroscopy, circular dichroism (CD), and computational modeling studies. The resulting Zn²⁺/netropsin:3'FdU complex had very high thermal stability with aspects of the complex intact at 85 °C, conditions that result in complete dissociation of Mg²⁺ complexes. CD and ¹⁹F NMR spectroscopy were consistent with Zn²⁺ binding in the major groove of the DNA duplex and utilizing F5 and O4 of consecutive FdU nucleotides as ligands with FdU nucleotides hemi-deprotonated in the complex. Netropsin is bound in the minor groove of the DNA duplex based upon 2D NOESY data demonstrating contacts between AH2 1H and netropsin 1H resonances. The Zn²⁺/netropsin:3'FdU complex displayed increased cytotoxicity towards PC3 prostate cancer (PCa) cells relative to the constituent components or separate complexes (e.g. Zn²⁺:3'FdU) indicating that this new structural motif may be therapeutically useful for PCa treatment.

Introduction

The coordination of metals by nucleic acids has important implications for biology, with metals such as Cd²⁺ and Ni²⁺ imparting toxicity and carcinogenesis (Bal et al. 2003; Franzle & Markert 2003; Beyersmann & Hartwig 2008; Hartwig 2010) while metals such as K⁺ and Mg²⁺ (Broxson et al., 2011; Yang & Hayes 2011) play pivotal roles in nucleic acid structure and stability. Zn²⁺ plays a key role in cellular homeostasis in part through its coordination by Zn²⁺-finger peptidic motifs that are important regulators of protein-protein (Gamsjaeger et al., 2007) and protein-nucleic acid (Elrod-Erickson et al., 1996) recognition. An intriguing observation is that Zn²⁺ levels decrease dramatically during prostate cancer (PCa) carcinogenesis with normal prostate characteristically displaying the highest Zn²⁺ levels for any soft tissue in the human body (Kelleher et al., 2011) while PCa displays low levels of Zn²⁺ (Christudoss et al., 2011). Exogenous Zn²⁺ is cytotoxic towards PCa cells and Zn²⁺-treatment sensitizes PCa cells to chemotherapy (Uzzo et al. 2002).

5-Fluoro-2'-deoxyuridine (FdU) is a non-native thymidine analog that has anti-cancer activity as a consequence of metabolism to the thymidylate synthase (TS) inhibitory metabolite FdUMP (Gmeiner 2005) and the incorporation of the nucleotide triphosphate FdUTP into DNA that results in poisoning of DNA Topoisomerase 1 (Liao et al. 2005;

* Address Correspondence to this author: Phone (336) 716-6216 Fax: (336) 716-0255 bgmeiner@wfubmc.edu.

Gmeiner et al., 2010; Gmeiner et al., 2011; Pardee et al., 2012). FdU is readily cleaved by glycosidase activities *in vivo* to 5FU and 5FU is widely used to treat diverse malignancies imparting a significant survival advantage for treatment of colorectal cancer. 5FU is inefficiently converted to FdUMP and FdUTP, however, and thus there is a need for alternative approaches to deliver DNA-directed fluoropyrimidine (FP) drugs. Our laboratory has pioneered the use of DNA polymers of FdUMP with FdUMP[10] showing particularly strong cytotoxicity and a unique spectrum of activity in the NCI 60 cell line screen (Gmeiner et al., 2010).

The chemosensitization of PCa cells by Zn^{2+} raises the possibility that Zn^{2+} chelates of FdU-oligodeoxynucleotides might be useful for treating advanced PCa. Zn^{2+} was reported to form specific complexes with FdU-substituted DNA (Lee, Latimer & Reid 1993) and coordination of Zn^{2+} by deprotonated FdU was proposed as the basis for specific complex formation. Zn^{2+} -DNA complexes formed under similar conditions were however, found to be the result of inter-strand aggregation (Spring & Clegg 2007). We recently reported that Zn^{2+} formed specific complexes with FdU-substituted DNA hairpins provided the DNA sequence contained several consecutive FdU nucleotides (Ghosh et al., 2011). Biophysical characterization and computational chemistry approaches revealed Zn^{2+} bound FdU-substituted DNA in the major groove. Zn^{2+} was bound in a trigonal bipyramidal geometry with F5 from one FdU and O4 from an adjacent FdU serving as axial ligands for Zn^{2+} coordination. Zn^{2+} complexation required hemi-deprotonation of FdU nucleotides with F5 being activated for coordination by deprotonation. Hemi-deprotonation of the complex occurs near physiological pH consistent with the pK_A for FdU (~7.6).

The occupancy of the major groove of DNA through Zn^{2+} complexation raises the possibility that Zn^{2+} :DNA complexes could be further stabilized by binding of ligands in the minor groove. Minor groove binding may impart greater thermodynamic stability to the Zn^{2+} :DNA complex and could potentially result in increased uptake into tumor cells through reduced charge density since minor groove binding ligands (Nelson et al., 2007), such as netropsin (Lewis et al. 2011), carry positive charges that partially offset the highly negative charge of the phosphodiester backbone of DNA. In this work, we demonstrate that netropsin and Zn^{2+} simultaneously occupy the minor and major groove in the DNA hairpin 3'FdU that includes 10 consecutive FdU nucleotides at the 3'-terminus (Fig. 1). CD and NMR spectroscopy indicate the binding of Zn^{2+} occurs via a similar structural motif as occurs for the Zn^{2+} :DNA complex while 2D NMR spectroscopy verifies that netropsin binds in the minor groove formed from A-FdU base pairs similar to the binding motif established for A-T base pairs. The resulting Zn^{2+} /netropsin:3'FdU complex is highly cytotoxic towards PCa cells consistent with this unique structural motif being useful for PCa treatment.

Methods

Formation of Zn^{2+} :DNA Complexes

Intramolecular hairpins were formed using 1 mM DNA in dH_2O by heating to 80°C for 5 min followed by rapid cooling. Native gel electrophoresis as described in the previous studies confirmed intramolecular DNA hairpin formation (Ghosh et al. 2011). Zn^{2+} /netropsin:3'FdU (or Mg^{2+}) complexes were formed by incubating 100 μ M DNA with 0 – 250 μ M netropsin and 0 to 5 mM $ZnCl_2$ (or $MgCl_2$) in M-DNA buffer (20 mM sodium cacodylate, pH 8.0, 10 mM NaCl) for 1 hour at room temperature. The formation of specific Zn^{2+} /netropsin:3'FdU trimolecular complexes was evaluated using an EtBr exclusion fluorescence assay as previously described (Ghosh et al. 2011). This assay was performed by combining 20 μ L of the above described trimolecular complex with 80 μ L of EtBr solution (10 mM NaCl, 20 mM sodium cacodylate, pH 8.0, + 25 μ M EtBr) followed by incubation for 30 min at room temperature in a 96-well clear bottom, black plate (Costar).

The final 3'FdU concentration was 20 μM and divalent metal and netropsin concentration ranged from 0 to 1 mM and 0 to 50 μM respectively. EtBr fluorescence was detected using a Typhoon-9210 variable mode imager with Green laser (532nm) excitation and 610 nm emission filter (with 500 PMT set). Fluorescence intensity was quantified with the software ImageQuant5.2 and graphed vs netropsin concentration.

Analytical Characterization of DNA:Zn²⁺ Complexes

Zn²⁺/netropsin:3'FdU and Mg²⁺/netropsin:3'FdU trimolecular complexes were analyzed by circular dichroism (CD) and NMR spectroscopy to characterize their structural features and any specific changes induced by netropsin and Zn²⁺ (or Mg²⁺). 300 μL of 20 μM solution of DNA with 0, 25, 50 μM netropsin and 0, 0.5 mM Zn²⁺ (or Mg²⁺) were prepared in M-DNA buffer as described above and used to obtain the CD spectrum. An Aviv CD spectrometer was used to scan the region from 210 – 350 nm at a rate of 0.5 nm/sec. Temperature was controlled at 20 °C. For temperature-dependent CD studies, temperature was increased from room temperature to 37, 55, 70, 85 °C respectively and allowed to equilibrate for an additional minute then spectra were acquired at the above mentioned temperature. ¹H, ¹⁹F, and ³¹P NMR spectroscopy were used to investigate specific structural perturbations induced by netropsin and Zn²⁺ to the 3'-FdU hairpin. For the NMR analysis, 500 μL of 0.5 mM 3'FdU in M-DNA buffer modified to contain 50 mM sodium cacodylate were used. NMR samples contained 0.5 mM netropsin and either no metal, 5 mM Zn²⁺ or 5 mM Mg²⁺. 10% D₂O was included to maintain lock. The final pH of the samples was adjusted to 8 (+/- 0.1 pH unit).

¹H NMR spectra were collected using a Bruker Avance DRX 600 MHz spectrometer with a TXI Cryoprobe at room temperature using the WATERGATE sequence with a 3-9-19 refocusing pulse. Nulls were set at +/- 18.1 ppm. Spectra were acquired with a spectral width of 33.3 ppm, 8192 total data points, and 256 scans. Two-dimensional NOESY experiments were acquired at 25 °C using a 3 s relaxation delay, a 200 ms mixing time, 48 scans per increment, 4096 $\times$ 256 complex points, and 3-9-19 WATERGATE solvent suppression (Sklenar et al., 1993). Data were processed using NMRPipe (Delaglio et al. 1995).

¹⁹F and ³¹P NMR spectra were acquired using a Bruker 300 MHz NMR instrument with QNP probe at room temperature using inverse gated proton decoupling. ¹⁹F spectra were acquired with a spectral width of 100 ppm, 7000 total data points and 1000 scans. ³¹P spectra were acquired with a spectral width of 400 ppm, 65536 total data points and 1000 scans. Both ¹⁹F and ³¹P spectra were acquired with concentric internal standard capillaries (Wilmad) 85% H₃PO₄ (δ = 0 ppm) or 5 mM aqueous solution of KF (δ = 125.3 ppm). NMR spectra were processed with Mestrec 4.995 software and referenced to internal standards.

T_m analysis

Temperature-dependent melting (T_m) of the 3'FdU DNA hairpin complexed or not complexed with Zn²⁺/Mg²⁺ and/or netropsin was determined using UV absorption spectroscopy at 260 nm. 400 μL of a 2.5 μM solution of the annealed hairpins in M-DNA buffer in the presence of 5 μM netropsin and 0.5 mM Zn²⁺ (or Mg²⁺) were analyzed using a Beckman Coulter DU-800 UV-Vis spectrophotometer in the temperature region 20 – 85 °C. Temperature was increased at a rate of 0.5 °C/min. Melting temperatures were estimated from the percent increase in hyperchromicity.

Cytotoxicity of FdU Hairpins and Zn²⁺ Complexes

The cytotoxicity of the 3'FdU-netropsin-Zn²⁺ trimolecular complexes was determined using a modified clonogenic assay with an MTT read-out. PC3 prostate cancer cells were seeded

in 24 well plates at a cell density of 1000 cells/well with 500 μ L complete media (RPMI + 10%FBS + 1% Penicillin & Streptomycin (pen/strep)). After 24 hours, media was removed and cells were washed twice with 500 μ L PBS followed by addition of 500 μ L serum and pen-strep free RPMI (pH-8.0). The 3'FdU-netropsin-Zn²⁺ complex was formed by incubating 10 μ M 3'FdU hairpin with 0.2 mM netropsin and 0.5 mM Zn²⁺ in serum- and antibiotic-free RPMI (pH-8.0) at room temperature for 1 hour. The cells were then treated with the above complex and incubated at 37 °C in a CO₂ incubator. After 24 hours, media was replaced with fresh, complete media and incubated at 37°C for a total of 14 days with media changes every 2 days. Cell viability was then assessed using an MTS assay. Wells were washed with sterile PBS followed by addition of 250 μ L fresh complete media. Then 25 μ L CellTiter 96 AQueous One Solution Cell Proliferation Assay reagent (Promega) was added to each well and incubated at 37 °C for 3 hours in a CO₂ incubator. 200 μ L of the resultant solution from each well were transferred to a transparent 96-well plate and the absorbance was measured at 490nm using Spectra Max-340PC microplate reader. All experiments were done in triplicate. Each set of data (net absorbance) were then expressed as a percentage, considering the no treatment group as 100%.

Computational Modeling

The initial models for the Zn²⁺ complexes were derived from coordinates for six consecutive base pairs and netropsin extracted from the NMR solution structure (pdb 1Z8V) (Van Hecke et al., 2005). Conversion to A-FdU was performed by first rebuilding the A-T DNA chain using the coordinate building tools within CHARMM (Brooks et al. 2009) on the original pdb backbone, and then deleting the thymidine methyl group and replacement with fluorine. In keeping with our previous work (Ghosh et al. 2011), every other FdU base nitrogen was deprotonated to form the hemi-protonated states. Also based on our previous work, (Ghosh et al. 2011) Zn²⁺ complexes with three water molecules were added to the DNA/netropsin complex. This was attempted with six, three and two complexes spaced evenly, however, only the two Zn²⁺/three water complexes formed stable complexes with netropsin/DNA. Geometry optimizations were performed on the entire complex, with Mg²⁺ counterions added in the vicinity of the backbone phosphorus. The structures were geometry-optimized with Gaussian 03 using PM3 in Cartesian coordinates (Ghosh et al. 2011). Post-optimization, single-point calculations were performed with a minimal basis set (STO-3g*) for an Natural Bond Orbital analysis (Frisch et al., 2004) from which the Wieburg bond orders were extracted. The entire complex was allowed to be flexible with a total RMSD of 1.17 Å from the start of computations.

RESULTS

Stabilization of Zn²⁺:DNA Complexes through Netropsin Binding

We have previously established that DNA sequences consisting, in part, of consecutive dAFdU base pairs, such as the DNA hairpin sequence 3'FdU (Fig. 1) form highly stable complexes with Zn²⁺. Zn²⁺ complex formation occurs via major groove binding to the DNA duplex and requires hemi-deprotonation of the FdU imino hydrogens. As netropsin is known to bind in the minor groove of duplex DNA selectively at runs of consecutive dA-dT base pairs, we sought to determine if netropsin would also bind to consecutive dA-FdU base pairs and, if so, to determine to what extent netropsin binding (likely in the minor groove) would cooperatively stabilize Zn²⁺-binding in the major groove of the 3'-FdU sequence. The minor groove atomic configuration for FdU-substituted DNA sequences, such as the 3'FdU DNA hairpin, is identical to that for DNA sequences consisting of dA-dT base pairs making it likely that netropsin would bind in a similar manner. Further, netropsin binding would be expected to reduce base pair dynamics for DNA complexes (Zimmer et al., 1971). This reduced flexibility could result in cooperative enhancement of Zn²⁺-binding in the major

groove if the preferred geometries for DNA in the netropsin complex and the Zn^{2+} complex were similar.

The propensity for netropsin binding to alter the formation and stability of Zn^{2+} complexes with the hairpin 3'FdU were studied using EtBr exclusion assays (Fig. 1c,d and Supplementary Fig. 1), native gel electrophoresis (Supplementary Fig. 2) and UV (Supplementary Fig. S3) and CD (Fig. 2) thermal melting studies. We have previously shown that EtBr intercalation into 3'FdU is inhibited by Zn^{2+} complexation (Ghosh et al. 2011), but is not blocked by Mg^{2+} binding. Netropsin binding alone inhibited EtBr intercalation consistent with netropsin binding 3'FdU and stabilizing the duplex in a manner that prevents EtBr intercalation. Consistent with our previous results, Zn^{2+} -binding alone inhibited EtBr intercalation. Interestingly, the combination of Zn^{2+} and netropsin was more effective at inhibiting EtBr intercalation than either agent alone, consistent with cooperativity in the binding of these moieties to distinct sites on the DNA duplex (Fig. 1c,d).

The thermal stability of the 3'FdU complex with Zn^{2+} and netropsin (Zn^{2+} /netropsin:3'FdU) was further investigated using UV hyperchromicity (Supplementary Fig. S3) and temperature-dependent CD spectroscopy (Fig. 2). We had previously demonstrated that Zn^{2+} complexation resulted in a substantial increase in thermal stability for 3'FdU while Mg^{2+} binding had a more moderate stabilizing effect (Ghosh et al. 2011). UV hyperchromicity measurements revealed that the sigmoidal-shaped curve typically observed in such experiments did not occur for Zn^{2+} /netropsin:3'FdU although such sigmoidal curves were detected for the Mg^{2+} /netropsin:3'FdU complex and the netropsin:3'FdU complex (Supplementary Fig. 2). The overall increase in absorbance (260 nm) for both the Mg^{2+} /netropsin:3'FdU complex and the netropsin:3'FdU complex was the 20-25% that is typical for melting duplex DNA and both complexes had similar T_m values. In contrast, Zn^{2+} /netropsin:3'FdU displayed a maximum increased absorbance of about 15% indicating strand-dissociation was not complete, even at temperatures above 80 °C. These results indicated that Zn^{2+} and netropsin may cooperatively stabilize 3'FdU and that the Zn^{2+} /netropsin:3'FdU complex likely undergoes stepwise dissociation with the complex remaining incompletely dissociated even at elevated temperatures.

CD Spectral Analysis

The structure and stability of Zn^{2+} /netropsin:3'FdU was further evaluated by CD spectral analysis, including temperature-dependent studies, to determine to what extent Zn^{2+} and netropsin binding, both singly and in combination, alters DNA structure and to gain further insight into the thermal stability of the complex (Fig. 2) and the cooperativity of ligand binding (Supplementary Fig. 3). CD spectra for 3'FdU:netropsin revealed highly specific alterations to the structure and stability of the complex were induced by Zn^{2+} (Fig. 2a,c), but not Mg^{2+} (Fig. 2b,d). The effects of netropsin binding to 3'FdU were similar to those observed previously for binding to A-T rich DNA duplex sequences and involve a slight red-shift for the maximal positive ellipticity (280 → 285 nm), a sharp new maximum at 260 nm, and a broad new maximum at approximately 320 nm (Fig. 2). The red-shift for the maximal ellipticity and the new maxima at 260 nm likely involve slight structural alterations for the A-FdU base pairs to accommodate netropsin binding in the minor groove while the new 320 nm maxima reflect the binding of N-methyl pyrrole moieties of netropsin in the minor groove of 3'FdU (Zimmer et al. 1979). The cooperativity of Zn^{2+} and netropsin for inducing structural alterations in DNA was assessed by analysis of ligand-dependent changes to the CD spectra (Supplementary Table 1 and Supplementary Fig. 3). While netropsin alone or Zn^{2+} alone induced moderate change in ellipticity, the combination of Zn^{2+} and netropsin induced substantial changes consistent with cooperativity in altering DNA structure (Supplementary Fig. 3).

The combined effects of Zn^{2+} (and Mg^{2+}) and netropsin to complex stability were further investigated by temperature-dependent CD spectroscopy. While the temperature sensitivity for CD spectra of the $\text{Mg}^{2+}/3'\text{FdU}$ complex were nearly indistinguishable from netropsin: $3'\text{FdU}$ indicating Mg^{2+} had little effect on the thermal stability of this complex the 260 nm maxima for the $\text{Zn}^{2+}/\text{netropsin}:3'\text{FdU}$ complex was attenuated by only ~10% upon increasing the temperature from 37 to 70 °C and the intensity for this maxima was reduced only about 30% upon increasing the temperature to 85 °C. These results complement the UV melting studies that demonstrated incomplete melting for the $\text{Zn}^{2+}/\text{netropsin}:3'\text{FdU}$ complex, based on percent change in UV absorption, at 70 °C (Supplementary Fig. 4) and are consistent with Zn^{2+} and netropsin binding cooperatively to $3'\text{FdU}$ and resulting in formation of a highly stabilized complex.

NMR Spectroscopy

The interactions of Zn^{2+} and netropsin with $3'\text{FdU}$ in the context of the $\text{Zn}^{2+}/\text{netropsin}:3'\text{FdU}$ complex were investigated using NMR spectroscopy. Our previous studies with the $\text{Zn}^{2+}/3'\text{FdU}$ complex revealed significant downfield shifts for approximately one-half of the ^{19}F resonances relative to $3'\text{FdU}$ in the absence of Zn^{2+} (Ghosh et al. 2011). Computational and modeling studies indicated that F served as one ligand for Zn^{2+} coordination in the $\text{Zn}^{2+}/3'\text{FdU}$ complex and that F was activated through deprotonation of the FdU imino 1H which occurred for about one-half of FdU nucleotides under the experimental conditions used. ^{19}F NMR spectra for $\text{Zn}^{2+}/\text{netropsin}:3'\text{FdU}$ revealed similar ^{19}F downfield shifts occurred for this complex consistent with Zn^{2+} coordination involving F in this complex, as well (Supplementary Fig. 5a). In contrast, no significant changes were noted in the ^{19}F NMR spectrum for the $\text{Mg}^{2+}/\text{netropsin}:3'\text{FdU}$ complex and netropsin alone did not induce such changes to the ^{19}F spectrum (Supplementary Fig. 5a). ^{31}P NMR spectra indicated no significant change upon Zn^{2+} and/or netropsin binding to $3'\text{FdU}$ although changes were detected for $\text{Mg}^{2+}/\text{netropsin}$ consistent with Mg^{2+} , but not Zn^{2+} , interacting with the phosphodiester backbone of $3'\text{FdU}$ (Supplementary Fig. 5b).

The interactions of Zn^{2+} and netropsin with $3'\text{FdU}$ were further analyzed by 1D and 2D ^1H NMR spectroscopy. Imino ^1H resonances for FdU nucleotides were readily detected at characteristic frequencies for the $\text{Zn}^{2+}/\text{netropsin}:3'\text{FdU}$ complex consistent with our previous studies (Ghosh et al. 2011) indicating hemideprotonation, rather than full deprotonation, was required for complex formation (Supplementary Fig. 5c). 2D NOESY spectra were obtained to gain insight into the binding of netropsin to $3'\text{FdU}$ (Fig. 3). The binding of netropsin, or other putative minor groove binding ligands, at A-FdU (rather than A-T) base pairs had not been described previously, however our 2D NOESY studies demonstrate netropsin interacts with $3'\text{FdU}$ in the minor groove. NOESY crosspeaks were observed between resonances with chemical shifts consistent with an AH2 resonance assignment and those consistent with netropsin ^1H resonances (Fig. 3). NOESY crosspeaks from FdU imino ^1H to AH2 resonances confirmed Watson-Crick base pairing is maintained in the complex.

Cytotoxicity of $\text{Zn}^{2+}/\text{netropsin}:3'\text{FdU}$ to PC3 Cells

Our previous studies demonstrated that $3'\text{FdU}$ is cytotoxic to PC3 prostate cancer cells and that Zn^{2+} complexation increased the cytotoxic effects (Ghosh et al. 2011). In these studies, the $\text{Zn}^{2+}/3'\text{FdU}$ complex was delivered to cells using oligofectamine both to enhance cellular permeability and because the complex was labile under conditions used in the tissue-culture experiments. In light of the increased thermal stability for $\text{Zn}^{2+}/\text{netropsin}:3'\text{FdU}$ relative to the $\text{Zn}^{2+}/3'\text{FdU}$ complex we sought to determine if this complex might display increased cytotoxicity towards PCa cells. Cytotoxicity was determined using a modified clonogenic assay with an MTS assay readout (Ghosh et al. 2011). Under the

conditions used for these experiments the 3'FdU hairpin and the Zn^{2+} /3'FdU complex displayed similar cytotoxicity consistent with dissociation of Zn^{2+} , which had moderate cytotoxicity as a single agent (Fig. 4 and Supplementary Table 2). The netropsin:3'FdU complex displayed slightly lower cytotoxicity than 3'FdU, and netropsin as a single agent actually increased viability relative to control cells. These results indicate netropsin is growth-stimulatory towards PCa cells, a surprising finding in light of the antitumor/antibiotic activity described previously for netropsin (Soderlind et al. 1999). The Zn^{2+} /netropsin:3'FdU complex displayed enhanced cytotoxic effects relative to the individual components (Zn^{2+} , netropsin, 3'-FdU) and relative to Zn^{2+} /3'FdU complex. In light of the proliferative effects of netropsin, these findings demonstrate that netropsin is likely enhancing the cytotoxicity of the Zn^{2+} /netropsin:3'FdU complex by stabilizing the complex and possibly through enhanced cellular uptake.

Computational Modeling of Zn^{2+} /netropsin:3'FdU

The preferred geometry for Zn^{2+} binding to DNA sequences containing six FdU nucleotides with netropsin was calculated using Gaussian 03 (Frisch et al., 2004). Specifically, the coordinates for six consecutive base pairs were extracted from the structure for a DNA bound to netropsin (rcsb ID; 178V (Van Hecke et al. 2005)). The DNA chain was truncated to include just six base pairs along netropsin and computationally mutated with the sequence and building tools in CHARMM (Brooks et al. 2009) to an A-FdU sequence, but with the backbone provided from the original pdb file.

The geometry and charge distribution for the complex were optimized in an approximation to the 'biological pH state' in which every other FdU was ionized. Zn^{2+} complexes with three water molecules were added to the system in geometries mimicking that of our previous study. Two, three, and six Zn^{2+} complexes were added for different calculations, but only a two Zn^{2+} system was found to stably bind. The overall geometry and charge distribution were optimized for minimal energy as described in the methods. The structure of each binding site with the Zn^{2+} interacting with adjacent nucleotide bases is shown in Fig. 5. Stereo-views of the binding sites are presented in Supplementary Fig. 6. As expected from our previous study (Ghosh et al. 2011), in each site the Zn^{2+} is located in the major groove with simultaneous interactions with the O4 carbonyl oxygen of the 3'-terminal FdU and F5 of the 5'-terminal FdU, which is deprotonated in the low energy structure. However, in site 2, the Zn^{2+} also interacts with the O4 of the 5' FdU.

More specifically, in site one, the Zn^{2+} is coordinated by three water molecules with an average distance of 2.13 Å to the oxygen atoms in the water molecules and with an average bond order of 0.314 (Supplementary Table 3). In site one, the Zn^{2+} is also coordinated by the above-mentioned F with a distance of 3.19 Å, a bond order of 0.006, and is coordinated by the previously mentioned O4 with a distance of 1.9 Å with a partial covalent bond (bond order 0.525 – Supplementary Table 3). This coordination is similar to that in our dinucleotide model where the Zn^{2+} had four electrostatic interactions with three water molecules and an F5, and a partial covalent bond with O4 (Supplementary Table 3). The interaction with the Zn^{2+} is more covalent, but the general mode of interaction is robust between the two models.

In the second site, much of the coordination is preserved, however, there is a key difference in the interaction with the 5'-nucleotide; rather than just an electrostatic interaction with the F5, there is also a close, partially covalent interaction with the O4 of that nucleotide. While there are still three water molecules coordinating the second Zn^{2+} , with an average O- Zn^{2+} distance of 2.1 Å and an average bond order of 0.273, and the F5 interactions is still predominately electrostatic (3.03 Å with a bond order of 0.0186), the interactions with the O4 atoms are different for this site. There are two partial covalent bonds to the two different

O4 atoms with bond orders of 0.32, and 0.5619. The lower bond order corresponds to the short distance, 1.92 Å, whereas the other O4 is further away at 2.01 Å. These findings indicate that there is a combination of electrostatic and covalent bonding with the O4 of each of the two flanking nucleotides with the Zn^{2+} in the second binding site.

Discussion

The present work demonstrates that Zn^{2+} and netropsin can simultaneously bind DNA hairpin sequences containing consecutive FdU nucleotides with Zn^{2+} occupying the major groove and netropsin binding the minor groove of the DNA double helix. The evidence in support of this unique structural configuration includes CD spectral data which are consistent with perturbations to the DNA nucleobases occurring in a Zn^{2+} - and netropsin-dependent manner. The NMR spectral data also were consistent with the major groove binding motif for Zn^{2+} previously established based on NMR and computational chemistry data from our laboratory and with netropsin binding in the minor groove based upon NOE data between netropsin ^1H resonances and AH2 ^1H resonances in the minor groove of the 3'-FdU hairpin.

The simultaneous binding of netropsin and Zn^{2+} to 3'-FdU resulted in significant stabilization of the complex. The thermal stability of the Zn^{2+} /netropsin:3'-FdU complex could not be completely assessed by UV hyperchromicity measurements because complete strand-dissociation was not achieved, even for the highest temperatures used for these studies (~85 °C). Temperature-dependent CD data demonstrated that the complex remained largely intact at 70 °C and remained at least partially present at 85 °C. This high thermal stability was found to correlate with improved cytotoxicity consistent with the complex persisting under physiological conditions for times sufficient for cellular uptake. The improved stability for the Zn^{2+} /netropsin:3'-FdU may be useful for *in vivo* studies and result in increased circulation times for the intact complex relative to linear homopolymers of FdUMP, such as FdUMP[10] which has shown promise in pre-clinical studies for cancer treatment (Pardee et al. 2012).

The ability to simultaneously target the major and minor grooves of DNA with potentially cytotoxic moieties as well as to incorporate cytotoxic nucleotides into DNA provides an opportunity to create moderately-sized multi-modality DNA therapeutic complexes that are based on non-covalent chemistry. The present studies have demonstrated proof of principle using netropsin as the minor groove binding ligand with Zn^{2+} occupying the major groove. Somewhat surprisingly, netropsin alone was somewhat growth stimulatory to PCa cells in these studies however the approach demonstrated here is applicable to other minor groove binding drugs, of which there are many, several of which are likely to enhance the anti-tumor activity of 3'-FdU when delivered as non-covalent complexes. As our studies with the linear homopolymer, FdUMP[10] indicate that DNA delivery of cytotoxic nucleotides is likely to impact treatment of cancer, the approach demonstrated here using non-covalent chemistry to create multi-modality DNA complexes may also be very useful for cancer treatment.

The structural basis for the high thermal stability of the Zn^{2+} /netropsin:3'-FdU complex appears to arise from cooperative binding. Both netropsin-only and Zn^{2+} -only binding to 3'-FdU induce a new maxima at 260 nm in CD spectra, likely as a result of perturbations in the structure of the FdU nucleobases. This maxima is significantly enhanced upon simultaneous binding of Zn^{2+} and netropsin consistent with cooperativity among the two types of binding (Supplementary Figure 3). The NMR spectral data indicate that Zn^{2+} complexation for Zn^{2+} /netropsin:3'-FdU occurs in a manner similar to that observed previously for the Zn^{2+} /3'-FdU complex. While computational studies support a similar binding mode for Zn^{2+} as described

previously, they clarify the overall stoichiometry of Zn^{2+} binding (2 Zn^{2+} are bound per 6 FdU nucleotides) and also identify a second binding mode that represents a slight perturbation from the original binding motif. In this second binding mode both O4 and F5 from the deprotonated FdU form partial covalent bonds with Zn^{2+} . Both 2D NMR and computational modeling studies indicate netropsin binds 3 -FdU in the minor groove with similar interactions as have been described previously with A.T-rich DNA sequences.

In summary, we have prepared a unique non-covalent complex that includes DNA with incorporated cytotoxic nucleotides, a minor groove binding ligand with potential anti-tumor activity, and Zn^{2+} , a divalent metal that is cytotoxic to advanced PCa cells. The efficient use of both the minor and major groove of DNA enhances the cytotoxic potential of these complexes. The resulting complexes have very high thermal stability and display enhanced cytotoxicity towards PCa cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Sequence of 3'FdU - the DNA hairpin investigated in the present studies. (b) Computationally-derived model of hemi-deprotonated 3'FdU hairpin complexed with netropsin in the minor groove and Zn²⁺ in the major groove. (c) Zn²⁺, but not Mg²⁺ (d), enhances EtBr exclusion in combination with netropsin. EtBr exclusion data graphed as fluorescence intensity of EtBr bound to complex of 3'FdU to 0 – 1mM Zn²⁺ (or Mg²⁺) and 0 – 50µm netropsin. The fluorescence intensity of the complex is normalized to fluorescence intensity for EtBr bound to 3'FdU only.

Figure 2.

CD spectra demonstrate Zn^{2+} selectively induces a blue-shift and intensity enhancement for the absorption maximum (280 $\rightarrow$ 260 nm) in combination with netropsin upon binding to 3'FdU. Spectra were acquired in the presence or absence of Zn^{2+} / Mg^{2+} and netropsin at the indicated concentrations. (a) CD spectra of 3'FdU and 0, 25, 50 μM Netropsin and 0, 0.5 mM Zn^{2+} . (b) CD spectra of 3'FdU and 0, 25, 50 μM Netropsin and 0, 0.5 mM Mg^{2+} . (c) CD spectra of Zn^{2+} /netropsin:3'FdU at indicated temperatures. (d) CD spectra of Mg^{2+} /netropsin:3'FdU at indicated temperatures.

Figure 3. 2D NOESY NMR spectrum Zn^{2+} /netropsin:3'FdU. The region shown includes the aromatic ^1H resonances of adenine. The arrow denotes the location of imino ^1H to AH2 resonances with the particular AH2 indicated also giving an NOE to a netropsin exchangeable ^1H at 9.5 ppm consistent with netropsin binding 3'FdU in the minor groove.

Figure 4.

MTS assay readout of a modified clonogenic assay demonstrating enhanced cytotoxicity for Zn^{2+} /netropsin:3'FdU relative to the constituent components towards PC3 prostate cancer cells. Netropsin is slightly growth stimulatory towards PC3 cells while Zn^{2+} enhances the cytotoxicity of 3'FdU towards these cells.

Figure 5.

(a) Computationally-derived model for site 1 Zn^{2+} complexation to the hemideprotonated 3'FdU hairpin in combination with netropsin. Zn^{2+} is located in the major groove with a distorted trigonal bipyramidal geometry in which the fluorine from the deprotonated FdU is one axial ligand and O4 from the second FdU is the second axial ligand. Water molecules are the equatorial ligands. White dashed lines are used to illustrate the coordinating atoms. (b) Model for site 2 Zn^{2+} complexation depicting partial covalent interactions from O4 from consecutive FdU nucleotides with Zn^{2+} .