Published in final edited form as:

Mol Pharm. 2012 February 6; 9(2): 325–333. doi:10.1021/mp2005577.

Specific Inhibition of the transcription factor Ci by a Cobalt(III)-Schiff base-DNA conjugate

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Abstract

We describe the use of Co(III) Schiff base-DNA conjugates, a versatile class of research tools that target C2H2 transcription factors, to inhibit the Hedgehog (Hh) pathway. In developing mammalian embryos, Hh signaling is critical for the formation and development of many tissues and organs. Inappropriate activation of the Hedgehog (Hh) pathway has been implicated in a variety of cancers including medulloblastomas and basal cell carcinomas. It is well known that Hh regulates the activity of the Gli family of C2H2 zinc finger transcription factors in mammals. In *Drosophila* the function of the Gli proteins is performed by a single transcription factor with an identical DNA binding consensus sequence, Cubitus Interruptus (Ci). We have demonstrated previously that conjugation of a specific 17 base-pair oligonucleotide to a Co(III) Schiff base complex results in a targeted inhibitor of the Snail family C2H2 zinc finger transcription factors. Modification of the oligonucleotide sequence in the Co(III) Schiff base-DNA conjugate to that of Ci's consensus sequence (Co(III)-Ci) generates an equally selective inhibitor of Ci. Co(III)-Ci irreversibly binds the Ci zinc finger domain and prevents it from binding DNA *in vitro*. In a Ci responsive tissue culture reporter gene assay, Co(III)-Ci reduces the transcriptional activity of Ci in a concentration dependent manner. In addition, injection of wild-type *Drosophila* embryos with Co(III)-Ci phenocopies a Ci loss of function phenotype, demonstrating effectiveness *in vivo*. This study provides evidence that Co(III) Schiff base-DNA conjugates are a versatile class of specific and potent tools for studying zinc finger domain proteins and have potential applications as customizable anti-cancer therapeutics.

Keywords

Basal Cell Carcinoma; Cobalt chelate/Schiff base; Development; *Drosophila*; Hedgehog Signaling; Cubitus interruptus; Transcription Factor; Zinc Fingers

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Supporting Information

The supporting information contains detailed synthesis information for Co(III)-Ci and Co(III)- CiMut. It also contains control experiments verifying the identity of EMSA shifted bands CiZn and KrZn. Finally, it contains verification experiments PtcΔ136mut-Fluc is a Ci dependent luciferase readout of Ci activity. This information is available free of charge via the Internet at <http://pubs.acs.org>

Introduction

Zinc finger domains are compact globular protein structures in which Cys and/or His residues are coordinated to a Zn(II) ion, which is necessary for structure and function¹. Many zinc finger domains bind with DNA and RNA and are essential for the function and activity of the corresponding transcription factors²⁻⁴. Human Cys2His2 (C2H2) zinc finger domain proteins comprise the largest motif-containing-family with a predicted 4500 C2H2 zinc finger domains spread amongst 564 different proteins⁵. Current approaches to understand these proteins by blocking their function utilize antibodies and model organism mutant collections. However, mutant collections are incomplete and antibodies are not always functional both *in vivo* and *in vitro*. Therefore, new tools designed to investigate these proteins are highly desired.

The Co(III) containing Co(III) Schiff base complex (Co(III)-sb) (Figure 1) binds histidine residues via a dissociative ligand exchange of the labile axial ligands⁶⁻¹⁰. Co(III)-sb non-specifically and irreversibly inhibits protein activity by coordinating to important histidine residues in active sites and those critical to structure^{6, 8, 10}. In the context of a DNA binding C2H2 zinc finger, histidine binding by Co(III)-sb displaces the zinc ion and disrupts the structure of the zinc finger domain, preventing DNA binding⁷.

While the inhibitory action of unconjugated Co(III)-sb is not specific, the addition of a targeting moiety by conjugating DNA to Co(III)-sb significantly increased the potency and specificity for the transcription factor Sp1 *in vitro*⁷. This approach was extended to *in vivo* by conjugating Co(III)-sb to a 17-bp DNA sequence containing the Ebox consensus sequence of the Snail family of transcription factors (Co(III)-Ebox). Co(III)-Ebox is a specific and potent inhibitor of Snail family transcription factors in *Xenopus laevis*¹⁰. Co(III)-Ebox inhibition was 150 fold more effective than Co(III)-sb and was dependent upon the presence of both the Ebox consensus sequence as well as the Co(III)-sb¹⁰. We hypothesized that by substituting the DNA sequence conjugated to Co(III)-sb, it would be possible to change the target of the Co(III) Schiff base. The focus of this work was to vary the DNA targeting moiety and develop a specific and potent inhibitor of the Ci/Gli family of proteins, C2H2 zinc finger containing transcription factors regulated by the Hedgehog (Hh) signaling pathway¹¹.

Hedgehog signaling is essential for the growth and patterning of multiple tissues and organs during mammalian embryonic development¹²⁻¹⁵. Aberrant activation of Hh signaling drives the establishment and progression of a variety of tumors including basal cell carcinomas and medulloblastomas¹⁶⁻¹⁸. In mammals, Hh performs these functions through the regulation of the Gli family of C2H2 zinc finger transcription factors; in *Drosophila* the function is performed by a single C2H2 zinc finger transcription factor Cubitus Interruptus (Ci).

During tumorigenesis, Hh signaling can act in multiple manners. In medulloblastomas and basal cell carcinomas, activation of the Gli transcription factors directly drives tumor growth. In pancreatic and colon cancer, paracrine Hh signaling from tumor cells leads to activation of Hh target genes in the surrounding stroma^{19, 20}. Gli activation in the stroma leads to a proliferative signal sent back to the tumor, similar to what occurs in development^{21, 22}. While promising Hh anti-cancer therapeutics exist, (most notably GDC-0449 and HhAntag^{23, 24}), a number of limitations persist. These drugs are ineffective on tumors arising as a result of mutations in the Hh pathway downstream of the transmembrane protein Smo^{25, 26} and patient relapse has been observed by spontaneous mutation in Smo rendering the drug unable to bind and inhibit signaling²⁷. Young mice treated with HhAntag have permanent defects in bone growth including loss of proliferation in chondrocytes and premature fusion of the growth plate resulting in mice with truncated

limbs²⁸. It is important to develop inhibitors that target additional components of the Hh pathway. The Gli proteins are particularly appropriate as they represent the terminal step in the pathway and work identifying GANT61, an inhibitor of the Glis has shown promising results in xenograft tumor models²⁹.

Here, we show that changing the DNA targeting sequence of Co(III) Schiff base-DNA conjugates creates a specific and potent inhibitor of Ci. *Drosophila* was used as a model to thoroughly investigate the mechanism of Co(III) Schiff base-DNA conjugate action *in vitro* as well as *in vivo*. The optimal Gli binding consensus sequence (TGGG[T/A]GGTC) is known and 100% conserved with the *Drosophila* homologue Ci and therefore, results for Ci should directly translate to the Gli's^{30–33}. This study demonstrates the synthetic ease and versatility for creating an entire class of specific and potent Co(III) Schiff base-DNA conjugates. These conjugates can be utilized as experimental tools to study C2H2 zinc finger proteins and have potential applications as personalized anti-cancer therapeutics.

Materials and Methods

Co(III)-Oligonucleotide Conjugates

Co(III)-Ci and Co(III)-CiMut were synthesized by coupling an oligonucleotide containing 3 phosphorothioate linkages (indicated by *) at both the 3' and 5' ends of both strands (Integrated DNA Technologies) to a Co(III)-Schiff base complex via a 6-carbon amino-terminated linker at the 5' end of one of the strands. To improve yields, the synthesis of Co(III)-sb as previously described was modified and verified (See Supporting Information Figures 1–4)¹⁰. The oligonucleotide sequences used are as follows:

Co(III)-Ci: 5'-C*T*A*CCTGGGTGGTC*T*C*T-3'

Co(III)-CiMut: 5'-C*T*A*CCTGGGTAGTC*T*C*T-3'

CiZn and KrZn Protein Extract

Drosophila Schneider 2 (S2) cells were obtained from the *Drosophila* Genomics Resource Center and grown as directed. Plasmids used for transfection were UAS-CiZn, which consists of amino acids 440–684 of Ci (the zinc finger domain includes 453–603)³⁴, UAS-KrZn in which the zinc finger domain of Kr (amino acids 214–363) was cloned out of the DGRC cDNA clone RE30918 using the primers: 5'-CATGAATTCATGAAGGATCCATCTCGCGACAAAA – 3' and CATCTCGAGCGCGGGAGTAGGCGGCGACTGGA, digested and ligated into the EcoRI/XhoI sites of pUAST, and pMT-Gal4^{35, 36}. S2 cells were seeded at 4.5×10^6 cells/25cm² flask dish and transfected using the Qiagen Effectene Transfection Reagent Kit (Qiagen) with a total of 1 µg of DNA (0.5 µg UAS construct 0.5 µg of pMT-Gal4) following the manufacturer's protocol. 12 hours later the cells were induced by adding CuSO₄ to a final concentration of 1mM. After 18 hours, the cells were centrifuged and resuspended in 330 µL EMSA Binding Buffer (20 mM Hepes, pH 7.6, 150 mM KCl, 3 mM MgCl₂, 0.5 mg/ml BSA, 10% glycerol and 0.2 mM ZnSO₄). The cells were lysed by freezing overnight at –80C, thawed on ice, aliquoted and stored at –20C.

Radiolabeled Probe Reactions

10 pmol of annealed oligonucleotide probes (IDT DNA) or Co(III)-Oligonucleotide Conjugates were radiolabeled on their 5' end(s) with ³²P ATP by T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's protocol. Unincorporated nucleotides were removed using ProbeQuant G-50 Micro Columns (GE Healthcare Life Sciences). To create a single labeled probe, one strand was phosphorylated before being

annealed to the unlabeled reverse complement. The oligonucleotide sequences are as follows:

Ci: 5'-CTACCTGGGTGGTCTCT-3'

CiMut: 5'-CTACCTGGGTAGTCTCT-3'

Kr: 5'-GGCGAGAACGGGTAAAGATC-3'

KrMut: 5'-GGCGAGAACAAATTAAGATC-3'

Electrophoretic Mobility Shift Assay

For the irreversibility assays, 5 μ L of protein extract, 1 μ L of salmon sperm DNA (1 mg/ml), and 12 μ L of binding buffer were mixed with 1 μ L 0.2 μ M radiolabeled Co(III)-Ci and incubated at room temperature for 3 hours. Either 1 μ L of binding buffer or 1 μ L 30 μ M cold competitor was added and incubated for 15 minutes. For standard assays, 5 μ L of protein extract, 1 μ L of salmon sperm DNA (1 mg/ml), and 12 μ L of binding buffer were mixed with 1 μ L 0.2 μ M Co(III)-Ci (or other cold competitor) and incubated at room temperature for 3 hours. 1 μ L of 0.2 μ M radiolabeled oligo probe was then added and allowed to bind for 15 minutes. Reactions were subsequently resolved on a TBE/acrylamide gel and imaged/quantified on a Storm 680 (GE Healthcare) phosphorimager using ImageQuant software. Shift intensities were corrected for individual lane background and normalized with no competitor shifts set to 100%. Statistical analysis was performed using a t-test.

Transfection and Luciferase Assay

Drosophila Schneider 2 R+ (S2R+) cells were obtained from the *Drosophila* Genomics Resource Center, seeded at 10^5 cells/well of a 24 well plate and transfected using the Qiagen Effectene Transfection Reagent Kit (Qiagen) according to the manufacturer's protocol using 30 ng pACT-RL (Renilla control), 150 ng or 75 ng Co(III) Schiff base-DNA conjugate, 100 ng pPAC-HACi(m1-4) and 100 ng of ptc Δ 136-FLuc or ptc Δ 136mut-FLuc^{36, 37}. Transfected cells were incubated for 15 hours, lysed and analyzed with the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocol. Results were normalized by dividing by Renilla expression and are reported as fold inductions over pPAC-HACi(m1-4) + ptc Δ 136mut-FLuc levels. Statistical analysis was performed on results using a t-test.

Drosophila Injections

Drosophila embryos, 0–45 minutes old, were dechorionated with bleach, desiccated for 10 minutes, lined up on a glue-coated microscope slide, covered in halocarbon oil and microinjected according standard *Drosophila* injection protocols³⁸. Injections were through the posterior end of the embryo and the injected material was deposited along the ventral surface of the embryo. Injected embryos were left on the slides in oil, placed in an 18 °C incubator and given 48 hours to recover and develop before washing in heptane to remove the halocarbon oil, squashing in lactic acid and imaging denticle patterning by phase contrast light microscopy. Statistical analysis was performed using Fisher's Exact Probability Test.

Results

Co(III)-Ci binds specifically and irreversibly to the Ci zinc finger domain

The Ci zinc finger domain (CiZn) is known to bind a nine bp consensus sequence with high affinity (Supporting Information Figure 5). To determine the effectiveness of a modified Co(III)-sb targeted to Ci, we coupled an oligonucleotide (Figure 1A) containing the Ci

binding sequence (TGGGTGGTC) flanked by phosphorothioate linkages at the terminal ends (indicated by a *, Figure 1A, C–D) to a Co(III)-sb complex (Figure 1B) to prepare Co(III)-Ci (Figure 1C). The resulting conjugate was radiolabeled with ^{32}P and incubated with extracts from S2 cells overexpressing CiZn. Reactions were analyzed by electromobility shift assays. Three biological replicates were performed; a representative gel is shown (Figure 2A) with combined results quantified (Figure 2B).

10 nM radiolabeled Co(III)-Ci was able to bind to CiZn (Figure 2A, Lane 3). With addition of 1500 nM (150X) cold competitor, 36 $\pm$ 2.4% of radiolabeled Co(III)-Ci remained bound (Figure 2A, lane 4), whereas the same concentration of cold competitor was able to completely eliminate binding of a 10 nM radiolabeled Ci binding site DNA probe (Figure 2A lanes 7–9). More Co(III)-Ci remained bound to CiZn than the Ci binding site probe (Figure 2B, 100% versus 48 $\pm$ 12%, $p = 0.016$) even in the absence of competitor. These divergent results show that coupling Co(III)-sb to the Ci binding site oligo has resulted in a transition metal conjugate capable of irreversibly binding to CiZn.

To determine whether Co(III)-Ci binding was acting in a sequence-dependent manner, we coupled a single base-pair mutation of the Ci binding sequence (TGGGTAGTC) to Co(III)-sb to create Co(III)-CiMut (Figure 1D). This single base-pair differences abrogated Co(III)-CiMut binding to CiZn (Figure 2A, lane 5), showing a sequence specificity requirement in the oligo to target Co(III)-sb to a zinc finger domain and providing support for a direct interaction between CiZn and the DNA moiety of Co(III)-Ci.

Co(III)-Ci prevents the Ci zinc finger domain from binding to DNA

Since Co(III)-Ci binds irreversibly to the CiZn domain, the ability of Co(III)-Ci to prevent CiZn binding to DNA was assayed. Co(III)-Ci was incubated with a CiZn-containing cell extract and a radiolabeled DNA probe containing the Ci consensus sequence (CTACCTGGGTGGTCTCT) was added to allow CiZn binding. The DNA-protein complex was analyzed by EMSA, imaged and quantified. Co(III)-Ci reduced the amount of CiZn bound to the radiolabeled DNA probe to 34.5 $\pm$ 6.8%, a significant reduction compared to baseline (Figure 3A, lanes 3 and 4, $p = 0.0035$). At the same concentration, neither unmodified Co(III)-sb (Figure 3A, lane 7) nor Co(III)-CiMut (Fig 3A, lane 5) were able to significantly inhibit CiZn from binding DNA. Additionally, the same concentration of the Ci binding site oligo alone had no effect on CiZn's ability to bind the radiolabeled DNA probe (Figure 3, lane 8). To obtain inhibition similar to that of Co(III)-Ci (Figure 3B) required a 100 fold excess concentration of cold Ci binding site oligo (Figure 3A, lane 9).

To continue the analysis of specificity, a Co(III) Schiff base-DNA conjugate targeting another transcription factor was studied. Co(III)-Ebox has an oligo coupled to Co(III)-sb that targets the Snail family of transcription factors and is able to prevent their binding to DNA ¹⁰. If Co(III)-Ebox targets only the Ebox-binding transcription factors then it should be ineffective at preventing CiZn from binding DNA. Indeed, Co(III)-Ci was able to inhibit CiZn binding in a concentration dependent manner, whereas Co(III)-Ebox had no effect (Figure 3D). Co(III)-Ci inhibited CiZn's DNA binding to 18.5 $\pm$ 14.7%, a significant reduction compared to Co(III)-Ebox that had no effect ($p < 0.05$).

Co(III)-Ci does not prevent the highly related transcription factor Krüppel from binding to DNA

The sequence of the oligo attached to Co(III)-Ci targets it specifically to the Ci zinc finger domain. We expected Co(III)-Ci would have no effect on the DNA binding ability of transcription factors that bind different sequences. The closely related protein Krüppel (Kr) is a transcription factor in the same family as Ci and has a known consensus sequence

(AACGGGTAA) that is distinct from that of Ci^{39–41}. The Kr zinc finger domain (KrZn) was overexpressed in S2 cells and a lysate was prepared. The KrZn lysate was incubated for 3 hours at room temperature with buffer or competitor and the radiolabeled Kr probe (GGCGAGAAACGGGTAAGATC) added. DNA-protein complexes were analyzed by EMSA. KrZn bound to the Kr probe and binding was abrogated by addition of the unlabeled Kr binding site oligo (Supporting Information Figure 6). Further, KrZn did not bind a radiolabeled KrMut probe (GGCGAGAAACAAATTAGATC) and was not hindered by addition of 150X cold KrMut oligos (Supporting Information Figure 6). A non-specific band was identified and not used in further analysis (Supporting Information Figure 6).

Importantly, Co(III)-Ci had no effect on KrZn's ability to bind its target probe. Cold competitors were incubated with KrZn extract for 3 hours, radiolabeled Kr probe was added for 15 minutes and DNA-protein complexes were analyzed by EMSA, imaged and quantified. 10 nM and 500 nM Co(III)-Ci had no significant effect on KrZn's ability to bind radiolabeled Kr probe (Figure 4A, lanes 2–4), nor did 10 nM and 500 nM cold Ci oligos (Figure 4A, lanes 5–6). Comparatively, only 39 ± 9% of probe remained bound when competed with 500 nM cold Kr oligos, representing a significant reduction relative to buffer control ($p = 0.007$). These results confirm that Co(III)-Ci does not prevent a highly related transcription factor from binding DNA. The oligo directs the Co(III) Schiff base-DNA conjugates to the target DNA-binding protein, providing specificity.

Co(III)-Ci reduces the activity of full length Ci

Co(III)-Ci irreversibly binds to Ci and prevents it from binding DNA. This action should abolish the transcriptional activity of full length Ci. To confirm this, we used a luciferase reporter gene assay in S2R+ cells to probe transcriptional activity of Ci. The *ptc* gene is an endogenous target of Hh signaling and fusing the *ptc* promoter to luciferase creates ptcΔ136-FLuc, a Hh responsive luciferase readout of Ci activity in cell culture^{36, 37, 42}.

S2R+ cells do not express Ci and do not have a Ci-dependent transcriptional response to Hh. However, they retain many of the intracellular pathway components that respond to Hh⁴³. To generate a constitutively-active luciferase response that is dependent on Ci, we overexpressed Ci(m1-4), a constitutively-active mutant form of Ci³⁶. Expression of Ci(m1-4) in the presence of ptcΔ136mut-FLuc, a *ptc* promoter with mutated Ci binding sites, had no effect on luciferase reporter gene activity (Figure 5A, lane 1). However, co-transfection of Ci(m1-4) with ptcΔ136-FLuc induced a significant increase in reporter expression as compared to either ptcΔ136-FLuc alone or ptcΔ136mut-FLuc + Ci(m1-4) (Figure 5A). Both ptcΔ136-FLuc and ptcΔ136mut-FLuc without Ci(m1-4) had similar basal levels of expression (Supporting Information Figure 7). Taken together, these results demonstrate that ptcΔ136-FLuc expression is dependent on the binding and activity of Ci(m1-4) in S2R+ cells.

Co(III)-Ci irreversibly binds and inhibits Ci, attenuating Ci(m1-4) activation of ptcΔ136-FLuc. Co-transfection of 150 ng Co(III)-Ci reduced the signal to 35% compared to buffer only (Figure 5, $p < 0.005$), whereas co-transfection of 150 ng Co(III)-Ebox, or Ci oligos did not result in significant reduction. Co-transfection of Co(III)-CiMut reduced the luciferase signal to 64% of buffer alone; however Co(III)-Ci reduction was significantly greater when compared to Co(III)-CiMut ($p = 0.039$). Co(III)-CiMut addition did not effect Ci(m1-4) activation of ptcΔ136-FLuc when the amount co-transfected was reduced to 75 ng, whereas 75 ng Co(III)-Ci was still able to significantly reduce the signal to 64% ($p < 0.005$).

Co(III)-Ci specifically inhibits Ci *in vivo*

We have shown that Co(III)-Ci has high specificity for Ci and provided evidence for minimal off-target effects, however these results were observed *in vitro*. Ci plays a major role in *Drosophila* development and helps pattern the segmentally repeating denticle belts on a developing *Drosophila* embryo (Figure 6A)^{32, 44, 45}. Genetic removal of Ci in *ci*⁹⁴ null mutant embryos causes a specific denticle patterning phenotype – the fusion of some of the denticle belts (Figure 6B, arrow). If Co(III)-Ci is able to bind specifically to Ci and inhibit Ci's ability to bind DNA, we expect treatment of *Drosophila* embryos with Co(III)-Ci will phenocopy a *ci* null. This was investigated by injecting embryos with Co(III)-Ci at the syncytial blastoderm stage prior to cellularization and allowing the embryos to develop for 48 hours. Embryos were imaged and scored for Ci phenotypes. Injection of 1 μ M Co(III)-Ci caused an elimination of naked cuticle and the merging of adjacent denticle belts, creating a localized Ci null phenotype (Figure 6D, arrow) in the region immediately surrounding the site of injection in 26% of embryos (8 of 31) whereas injection of 1 μ M Co(III)-CiMut had no effect (0 of 26) (Figure 6C). Co(III)-Ci caused a statistically significant phenotype versus buffer alone ($p = 0.03$) and versus Co(III)-CiMut ($p < 0.005$) (Figure 6E).

Embryos injected with Co(III)-Ci showed a localized Ci null phenotype. The question arises if this disruption in denticle belt patterning is due to specific inhibition of the Hh pathway, or could be explained by non-specific effects of Co(III)-Ci or perhaps even injection artifacts. To address this we turned to Hh null embryos. In *hh* null embryos, full length Ci is processed into the Ci repressor, which actively represses target genes, and consequentially, *hh* null embryos have a lawn of denticles on their ventral surface with no indication of segmentation, a phenotype much more severe than a *ci* null (Figure 7, A and B). Ci repressor is a truncation of full length Ci, with the same zinc finger domain, and binds the same DNA sequence as full length Ci and therefore should be targeted by Co(III)-Ci. Injection of Co(III)-Ci into a *hh* null mutant embryo should inactivate the Ci repressor, partially rescue the segmentation defect, and phenocopy a *ci* null. Injection of 1 μ M Co(III)-Ci was indeed able to restore *hh*^{-/-} embryos to a *ci* null phenotype (Figure 7C, 4 of 12 embryos) whereas 1 μ M Co(III)-CiMut was not (Figure 7D, $n = 18$), a significant difference (Figure 7E, $p = 0.018$).

Discussion

Coupling the Ci consensus binding sequence to Co(III)-sb resulted in a potent complex able to specifically inhibit Ci protein from binding DNA^{7, 10}. The Co(III)-Ci complex was able to inhibit Ci's DNA significantly better than either Co(III)-sb or Co(III)-Ebox. Mutating a single base-pair in the oligo component of Co(III)-Ci abrogated its ability to inhibit binding. Co(III)-Ci effects were shown to be specific, as it was not able to inhibit the DNA binding ability of a highly related C2H2 zinc finger transcription factor, Krüppel, presumably due to differences in the DNA binding consensus sequence. These results demonstrate that very slight modification of the attached oligo can have profound effects on the targeting of a Co(III) Schiff base-DNA conjugate.

We have demonstrated the effectiveness of the Co(III)-Ci complex both *in vitro* and *in vivo*. *Drosophila* denticle patterning is a well-described process governed by a wide variety of proteins, many of which are C2H2 zinc finger proteins and altering the function of any of these results in a characteristic patterning defect⁴⁴. Injection of Co(III)-Ci showed a merging of the denticle belts at the site of injection, a localized phenocopy of a *ci* null. This patterning defect is distinct from what would be expected if Co(III)-Ci were inhibiting a different DNA binding protein, such as Even-skipped or Krüppel⁴⁴. At the highest concentration of Co(III)-Ci injected (10 μ M), 36% of embryos showed a phenotype. We believe two major technical issues prevent us from increasing the percent affected. First, the

injection location is critical yet precise injection remains technically difficult. Indeed, multiple preliminary injections not directly at the ventral surface failed to induce a phenotype. Second, the timing of the experiment adds another technical issue. Embryos must be injected prior to cellularization. Yet in wild type embryos, denticle belt patterning does not occur until stage 11, nearly 5 hours later⁴⁶. Therefore, Co(III)-Ci has the potential to nonspecifically interact with proteins for 5 hours before carrying out its specific function.

It remained possible that the phenotype could be explained by a localized lethality caused by the Co(III)-Ci complex. *hh* null embryos have a more severe patterning phenotype than *ci* null embryos, due to constitutive Ci repressor formation. Injection of *hh* null embryos with Co(III)-Ci should rescue the *hh* null to that of a *ci* null and indeed that is what we found. Were the phenotype due solely to lethality or injection artifacts we would not expect injection of Co(III)-Ci to improve the phenotype. These experiments indicate that injection of Co(III)-Ci specifically abrogates Ci function *in vivo* and does so in a manner dependent on the sequence of the oligo attached to the Co(III) Schiff base-DNA conjugate.

Previous studies have shown that the mechanism of Co(III) Schiff base-DNA conjugate inhibition of zinc fingers involve the replacement of the zinc(II) ion with Co(III)-sb through histidine coordination at the axial sites of the complex^{7, 10}. This in turn disrupts the structure of the C2H2 zinc finger and irreversibly inhibits the zinc finger from binding DNA⁷. Co(III)-sb will non-specifically bind and inhibit all zinc finger containing proteins *in vivo*. Conjugation of a binding site DNA moiety to Co(III)-sb targets it towards a specific zinc finger protein that binds to the DNA. C2H2 zinc finger domains are particularly appropriate targets where the cobalt can displace the zinc(II) ion and collapse the zinc finger¹⁰.

The exact molecular interaction between Co(III)-sb and the zinc finger domain and the mechanism of structural perturbation remains elusive. Mass spectrometry studies are not straightforward; in the gas phase and ionizing conditions, Co(III)-sb is unstable due to redox propensities of the metal center. We are actively studying the interaction at the molecular level with structural investigations including extensive NMR, circular dichroism, and tandem mass spectrometry analysis. Further, we are using model peptides to obtain thermodynamic and kinetic parameters of the Co(III)-sb/His interaction with and without a targeting domain. A complete understanding of the inhibitory mechanism will allow us to develop and tune the Co(III)-Ci probe.

While the Co(III) Schiff base-DNA conjugates bind in a sequence specific manner, only a fraction (36%) are irreversibly bound after three hours (Figure 2A). This result is consistent with what has been previously seen with Co(III) Schiff base-DNA conjugates¹⁰. We believe this shows that the conjugate exhibits at least two binding modes. First, the attached oligo maintains a quick, reversible binding interaction to zinc finger domains, localizing the compound near the specific zinc finger domain. Second, we believe the Co(III)-sb portion of the conjugate retains a slower irreversible binding interaction with the zinc finger domain. It seems unlikely due to physical constraints the oligo and cobalt are simultaneously bound. The size and identity of the linker may be important and work is currently in progress investigating this as well as identifying the binding mode(s) of Co(III) Schiff base-DNA conjugates.

By altering the oligonucleotide sequence of the DNA targeting moiety in the Co(III) Schiff base-DNA conjugate, we have created an irreversible inhibitor of Ci. Utilizing their inhibitory action, Co(III) Schiff base-DNA conjugates can be applied as tools to the study the functions of zinc finger proteins. It should be possible to extend their range of application by using commercially available modifications to the ends of DNA, such as the

attachment of a fluorophore or biotin. Addition of a fluorophore could provide for *in vivo* imaging opportunities. Further, addition of biotin would allow for affinity chromatography, western blots and other immunoanalytical methods. This work demonstrates Co(III) Schiff base-DNA conjugates represent a versatile class of specific and potent tools available for studying zinc finger domain proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. Richard Carthew (Northwestern University) and Dr. Sarah Smolik (Oregon Health & Science University) for providing DNA constructs. We thank Lisa Manus and Tulay Atesin for contribution to the synthesis and purification of Co(III)-Ci and Co(III)-CiMut. We thank Sara Hurtado and Dr. Vince Gerbasi for helpful discussion and technical advice. Finally we thank Maggie Sledd and Erin Schrader for their helpful advice on the manuscript. This work was supported by the National Institute of Health under Award P50CA090386 and the Center for Cancer Nanotechnology Excellence (CCNE) initiative of the National Institutes of Health's National Cancer Institute under Award U54CA119341.

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Figure 1. Chemical structures of Co(III) Schiff base-DNA complexes utilized in the experiments. (A) Ci Oligos. (B) Co(III)-sb. (C) Co(III)-Ci. (D) Co(III)-CiMut.

Figure 2.

Co(III)-Ci irreversibly binds to the Ci zinc finger domain. (A) Lane 1. 10 nM ^{32}P radiolabeled Co(III)-Ci. Lane 2. 10 nM ^{32}P radiolabeled Co(III)-Ci incubated with control S2 extracts not expressing the Ci zinc finger domain. The Ci zinc finger domain was overexpressed in S2 cells, cells were lysed and incubated for 3 hours with either 1X ^{32}P radiolabeled Co(III)-Ci (lanes 3 and 4), 10 nM ^{32}P radiolabeled Co(III)-CiMut (lanes 5 and 6) or ^{32}P radiolabeled Ci probe (lanes 8 and 9). Then either buffer (A, lanes 3, 5 and 8) or 150X cold competitor (lanes 4, 6 and 9) was added to reactions for 15 minutes. Reactions were analyzed by EMSA on a 4% TBE/acrylamide gel and performed in triplicate. Representative gels are shown. (B) EMSAs were quantified using ImageQuant software. Error bars are one standard deviation. Final reaction concentrations are given.

Figure 3.

Co(III)-Ci prevents the Ci zinc finger domain from binding DNA in a specific and concentration-dependent manner. (A) Lane 1. 10 nM of a ^{32}P radiolabeled Ci probe. Lane 2. 10 nM of a ^{32}P radiolabeled Ci probe incubated for 15 minutes in an S2 cell extract not expressing the Ci zinc finger domain. The Ci zinc finger domain was overexpressed in S2 cells, cells were lysed and incubated for 3 hours with buffer (lane 3), 1X Co(III)-Ci (lane 4), 10 nM Co(III)-CiMut (lane 5), 10 nM Co(III)-Ebox (lane 6), or 10 nM Co(III)-sb (lanes 3–7) and 1X, 100X or 150X cold oligo competitor (lanes 8–10). Then 10 nM of a ^{32}P radiolabeled Ci probe was added to reactions for 15 minutes. Reactions were performed in triplicate (representative gel is shown) and the reactions were analyzed by EMSA as previously described. (C) Varying concentrations ranging from 0.1 nM to 10 nM of Co(III)-Ci or (D) Co(III)-Ebox were analyzed for their ability to prevent the Ci zinc finger domain from binding to 10 nM ^{32}P radiolabeled Ci probe as in (A). (B) and (E) Triplicate EMSAs were quantified using ImageQuant software. Error bars are one standard deviation, (* = $p < 0.05$). Final reaction concentrations are given.

Figure 4.

Co(III)-Ci is unable to inhibit the Krüppel zinc finger domain from binding DNA. (A) Lane 1. 10 nM of a ^{32}P radiolabeled Kr probe. The Kr zinc finger domain was overexpressed in S2 cells, cells were lysed and incubated for 3 hours with buffer (lane 2), 1X or 50X Co(III)-Ci (lanes 3 and 4), 1X or 50X Ci Cold Oligos (lanes 5 and 6) or 1X or 50X Kr Cold Oligos (lanes 7 and 8). Then 10 nM of a ^{32}P radiolabeled Kr probe was added to the reactions for 15 minutes and the reactions were analyzed by EMSA as previously described. Reactions were performed in triplicate (representative gel is shown). The relevant Kr specific shift is indicated by an arrow and (B) was quantified using ImageQuant software. The higher, non-specific band (see Supporting Information Fig. 6) was left out of the analysis. Error bars are one standard deviation. Final reaction concentrations are given.

Figure 5.

Co(III)-Ci prevents target gene activation by a full length, constitutively active Ci. S2R+ cells were transfected with a constitutively active form of Ci, Ci(m1-4) (all columns), either the Ci responsive ptcΔ136-Fluc luciferase vector (columns 2–8) or ptcΔ136mut-Fluc in which the Ci binding sites have been mutated (column 1), and 150ng of either Ci binding site oligos (column 3), Co(III)-Ebox (column 4), Co(III)-CiMut (column 5), Co(III)-Ci (column 6) or 75 ng of Co(III)-CiMut (column 7) or Co(III)-Ci (column 8). Cells were then incubated for 12 hours, lysed and assayed for luciferase activity. Experiments were performed in triplicate and error bars represent one standard deviation.

Figure 6.

Injection of Co(III)-Ci but not Co(III)-CiMut is able to phenocopy loss of *ci* function *in vivo*. *Drosophila* cuticle mounts showing denticle belt patterning of 48 hour old (A) wild-type and (B) *ci*⁹⁴ (null) embryos. Wild-type embryos between 0 and 45 minutes old were microinjected with 1 μ M (C) Co(III)-CiMut or (D) Co(III)-Ci, allowed to develop for 48 hours, mounted and imaged by phase contrast light microscopy. Arrow points to denticle belt fusion characteristic of a *ci* mutant (B) and (D). (E) Wild-type *Drosophila* embryos were injected with buffer (column 1), Ci oligos (column 2), 1 μ M Co(III)-CiMut (column 3), 1 μ M Co(III)-Ci (column 4), 10 μ M Co(III)-CiMut or 10 μ M Co(III)-Ci and allowed develop and prepared as previously described. Bars represent the percent of embryos that showed a denticle belt fusion. No other phenotypes were seen. (* = $p < 0.05$) Injected concentrations are given.

Figure 7.

Injection of Co(III)-Ci but not Co(III)-CiMut is able to partially rescue the effects of a genetic *hh* null *in vivo*. *hh* null embryos create Ci repressor (diagram A') whereas a *ci* null will not (B'). These different genotypes result in distinct phenotypes. (A) *Drosophila* cuticle mounts showing the denticle belt patterning of 48 hour old (A) *hh* null and (B) *ci*⁹⁴ (null) embryos. *hh* null embryos between 0 and 45 minutes old were microinjected with 1 μ M (C) Co(III)-CiMut or (D) Co(III)-Ci, allowed to develop, mounted and imaged as previously. Arrow points to separation and patterning between denticle belts present in the *ci* null but never present in a *hh* null. (E) Percent of embryos that showed a rescue of denticle belt patterning. (* = $p < 0.05$)