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Raphael Enoque Ferraz de Paiva

Gold(I,III) Complexes Designed for Selective Targeting and Inhibition of Zinc Finger Proteins

Doctoral Thesis accepted by
the University of Campinas, Campinas, Brazil

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The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" (I found it!) but "That's funny..."

—Isaac Asimov

The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher inspires

—William Arthur Ward

I dedicate this thesis to every teacher (in the most fundamental sense of the word) I had during my life. Luckily, I've had many great teachers.

Supervisor's Foreword

Raphael joined my group as an undergraduate student at the Institute of Chemistry of the Campinas State University (UNICAMP), Brazil, in 2010. He obtained his master's degree working in the synthesis and biological evaluation of silver and platinum complexes with anti-inflammatory agents. Raphael developed his Ph.D. project focusing on the development of gold(I,III) compounds for the inhibition of *zinc finger* proteins. This project was developed at UNICAMP with a one year exchange at VCU under the supervision of Prof. Nicholas Farrell.

The zinc finger family of proteins was explored as target in this work, in particular, the nucleocapsid protein (NCp7) from the human immunodeficiency virus (HIV-1). One of the most remarkable structural and functional characteristics NCp7 is the presence of two -Cys-X2-Cys-X4-His-X4-Cys- zinc finger (ZnF) domains. Inhibition of this proteins leads to the loss of viral infectivity, suggesting that NCp7 inhibitors could be developed as alternatives to the typical anti-retroviral therapies available today.

The broad contribution of this thesis was on the development of gold-based compounds for the inhibition of zinc finger proteins. Gold(I) compounds were developed using phosphine ligands as "carriers" and labile pyridine derivatives or chloride as co-ligands. Innovative approaches were used for evaluating the interaction of gold(I) compounds with zinc fingers. Travelling-wave ion mobility mass spectrometry (TWIM-MS) coupled with tandem MS proved to be an extremely powerful technique for detecting metallation sites in zinc fingers, as well as for identifying and separating conformers. X-ray absorption spectroscopy (XAS) was used for identifying changes in the coordination sphere and geometry of gold. In addition, gold(III) compounds were also developed. Stabilizing gold(III) represents an interesting challenge, as it is prone to reduction in the biological media. The organometallic compounds [Au(2-benzylpyridine)Cl_2] is particularly noteworthy. It was able to inhibit NCp7 through a *sui generis* mechanism based on a gold-catalyzed C-S bond formation.

Finally, the dedication and motivation of Dr. Raphael de Paiva are noteworthy. He was always willing to help his colleagues and ready for new challenges. His published works and conferences awards confirm his wonderful work and his dedication to science. It was a great honor to me to write about Dr. Raphael Enoque Ferraz de Paiva and I wish him success in his career.

Campinas, Brazil
July 2018

Prof. Pedro Paulo Corbi

Abstract

Many complementary approaches have been used to inactivate the HIV-1 nucleocapsid protein (NCp7) zinc finger for therapeutic use. The cysteine residues of NCp7 are some of the most nucleophilic of all zinc-bound thiolates found in proteins. Although it is considered a noble metal, gold has a very rich chemistry under the right conditions. It is capable of forming coordination compounds in both of its typical oxidation states (1+ and 3+). Gold(I) forms linear compounds, with coordination number 2, while gold(III) forms square planar compounds with coordination number 4. Electron-rich metals such as gold are particularly suitable for designing metal-based zinc ejectors because, as soft Lewis acid electrophiles, they have high affinity for Cys residues. Without the proper tridimensional folding granted by Zn coordination, NCp7 is inactivated and unable to further recognize specific nucleic acid sequences.

A series of Au(I) complexes with the general structure [Au(L)(PR_3)] (R = Et or Cy) was designed. The two aromatic residues in the structure of NCp7 (Phe16 and Trp37) are responsible for π-stacking with purine and pyrimidine bases found on RNA and DNA. The presence of these residues can be used for introducing an extra selectivity component in the designed compounds, as an aromatic L ligand can be used. We also examined for comparison the "standard" gold-phosphine compound auranofin which contains a thiosugar ligand coordinated to {Au(Et_3P)}. The nature of the phosphine and the nature of L affect both the reactivity with the C-terminal NCp7 ZnF2 and the "full" NCp7 as well as the final coordination sphere of Au once incorporated into the protein. In this reaction, the first step is the electrophilic attack of the Au(I)-phosphine compounds on the Zn-coordinated residues, forming a heterobimetallic {R_3PAu}-ZnF species. Two alternative pathways open up from here on. In the most typical pathway, after Zn displacement, the {R_3PAu} moiety can remain coordinated to a Cys residue. Afterwards, the phosphine is lost and a Cys-Au-Cys *gold finger* (AuF) is obtained. In this mechanism, compounds with slower reactivity such as [Au(dmap)(Et_3P)] allow us to probe the initial auration steps, with species such as {Et_3PAu}-apoNCp7 still being present. Longer incubation times or more reactive compounds such as the chloride precursors provide information on the final species, the gold finger itself. In an alternative pathway, observed for the model compound auranofin, the {R_3PAu} moiety coordinates to a His residue, and the final hypothetical AuF obtained

from this pathway has Cys-Au-His coordination sphere. The Au(I)-phosphine series also had its cytotoxic properties investigated. The compound $[Au(dmap)(Et_3P)]^+$ demonstrated a cytotoxic selectivity >50 towards a tumorigenic T lymphoblast cell line (CEM) in comparison to a normal cell line (HUVEC). The compound $[Au(dmap)(Et_3P)]^+$ caused apoptosis on the CEM cell line through a mechanism independent of p38 MAPK, as opposed to auranofin.

Au(III) compounds are typically not very stable under biological conditions since Au (III) can be easily reduced and it has fast ligand exchange rates. Despite the limitations, many recent Au(III)-containing compounds have been rationally designed by tailoring the ligands to stabilize Au(III). The Au(III) compounds evaluated so far as ZnF inhibitors undergo reduction to Au(I) with loss of all ligands, thus it is commonly accepted that the oxidation state of incorporated gold in AuFs is 1+. By handpicking the ligands, it is possible to fine tune the stability of Au(III) complexes, stabilizing the Au (III) oxidation state even in the presence of peptides with high cysteine content such as ZnFs. Here, we explored the Au(III)(C^N) motif based on the organometallic compound $[Au(2\text{-bnpy})Cl_2]$ (2-bnpy = deprotonated 2-benzylpyridine) in comparison to a series of Au(III) complexes with typical κ^2N,N' chelators (2,2'-bipyridine, 4,4'-dimethyl-2,2'-bipyridine and 1,10-phenanthroline). The organometallic compound $Au(2\text{-bnpy})Cl_2$ had a *sui generis* mechanism of zinc displacement, never reported before for any metal compound, that consists in the transfer of the 2-bnpy ligand to a Cys residue from the protein, leading to Au-catalyzed C-S coupling.

Determining the actual coordination sphere of Au in the AuF obtained by interacting Au(I) and Au(III) complexes with ZnF proteins is an interesting challenge, and for that purpose, we used two innovative approaches that allowed us to obtain structural information in solution. Travelling-Wave Ion Mobility (TWIM) coupled to Mass Spectrometry (MS) was used to evaluate the interaction of the Au (I) compound $[AuCl(Et_3P)]$ with two model ZnF proteins that differ on the Zn coordination sphere: NCp7 ZnF2 (Cys_3His) and the human transcription factor Sp1 ZnF3 (Cys_2His_2). Furthermore, X-Ray Absorption Spectroscopy (XAS) was used in a "dual-probe" approach to monitor oxidation state, coordination sphere changes and geometry changes of both Au and Zn for the interaction of two series of compounds. In the first series, $[AuCl(Et\text{-}_3P)]$ and auranofin were compared when interacting with NCp7 ZnF2 and Sp1 ZnF3. TWIM-MS and XAS data indicate that $[AuCl(Et_3P)]$ leads to the formation of a Cys-Au-His AuF when interacting with Sp1 ZnF3, as opposed to the $\{Et_3PAu\}$-F species (observed by XAS) that evolves to the Cys-Au-Cys AuF (observed by TWIM-MS) identified for the interaction with NCp7 ZnF2. Finally, XAS was used to compare the interaction of two $[Au(dien)L]^{n+}$ compounds (dien = diethylenetriamine; L = Cl, n = 2; L = dmap, n = 3) with the same two zinc fingers and it was demonstrated that $[Au(dien)(dmap)]^{3+}$ retains the AuN_4 coordination sphere and square planar geometry of Au(III) when interacting with NCp7 ZnF2, suggesting that the compound behaves as a non-covalent Zn ejector. MS data confirmed Zn displacement, and led to the identification of a non-covalent adduct between $[Au(dien)(dmap)]^{3+}$ and the full-length NCp7 ZnF, supporting the non-covalent displacement hypothesis.

Preface

Zinc finger proteins were discovered recently in 1982 [1]. The first representative of this class of proteins, referred to as Transcription Factor III (TFIII), was identified by the study of *Xenopus laevis* oocytes. Zn binding was confirmed by X-ray absorption spectroscopy (XAS) [2], an alternative technique to the classical single-crystal diffraction and NMR-based techniques. This research was pioneered by Prof. Aaron Klug who combined the zinc-binding information provided by XAS with the already known sequence of TFIII and came up with the idea of a finger-like structure, which established a new principle of nucleic acid recognition. Transcription factors are proteins involved in the process of converting, or transcribing, DNA into RNA. Transcription factors include a wide number of proteins, excluding RNA polymerase, that initiate and regulate the transcription of genes. One distinct feature of transcription factors is that they have DNA-binding domains that give them the ability to bind to specific sequences of DNA called enhancer or promoter sequences. Some transcription factors bind to a DNA promoter sequence near the transcription start site and help form the transcription initiation complex [3, 4]. Regulation of transcription is the most common form of gene control. The action of transcription factors allows for unique expression of each gene in different cell types and during development.

Among the plethora of zinc fingers that have been discovered and studied so far, we chose the nucleocapsid protein (NCp7) from the human immunodeficiency virus (HIV-1) as the main protein target for the work discussed in this Thesis. One of the most remarkable structural and functional characteristics NCp7 is the presence of two -Cys-X2-Cys-X4-His-X4-Cys- zinc finger (ZnF) domains, typically found on nucleic acid binding proteins. The presence of ZnF domains is highly conserved among retroviruses [5] and any mutation on the Zn-bound residues results in the loss of biological function [6, 7]. On the infectious HIV-1, NCp7 appears closely and strongly associated to RNA on the viral core [8]. The two major functions of NCp7 are RNA binding and viral encapsidation, but new evidence also suggests that NCp7 has a role in some other processes such as RNA dimerization, Gag-Gag interactions, membrane binding, reverse transcription and stabilization of the pre-integration protein complex [5]. Inhibition of NCp7 makes the virus ineffective,

so the development of NCp7 inhibitors represents an interesting alternative to the reverse transcriptase and proteases inhibition typically explored for HIV infection treatment.

The human transcription factor protein Sp1 [also known as specificity factor (1)] was also investigated as a target, with the purpose of comparing the more reactive Cys_2His_2 ZnF motif with the Cys_3His motif found in the structure of NCp7. Sp1 *per se* is also an interesting *zinc finger* target, as it is overexpressed in many cancer and it is associated with poor prognosis. Sp1's function is complex, both activating and suppressing genes essential for cancer development and tumor suppressors, in addition to genes regulating cellular proliferation, differentiation, DNA damage response, apoptosis and angiogenesis [9]. The coordination sphere has also a direct effect on the zinc affinity of the protein. NCp7's Cys_3His motif was shown to bind zinc more tightly than Sp1's Cys_2His motif [10, 11].

Many complementary approaches have been used to inactivate the NCp7 zinc fingers for therapeutic use [12, 13]. Considering the zinc center as a target, an interesting suggestion to approach selectivity is that, based on the Lewis acid considerations for different zinc coordination spheres, zinc chelators may be effective for zinc coordination spheres with a labile catalytic site whereas electrophilic attack of zinc-bound thiolate may be more favored in cases of structural zinc where there are at least 2 Cys residues in the coordination sphere [14]. This hypothesis to some extent reflects the historical situation where early attempts to inactivate the zinc center used compounds such as S-acyl 2-mercaptobenzamide thioesters [15, 16]. The cysteine residues on the NCp7 are some of the most nucleophilic of all zinc-bound thiolates in proteins [17, 18]. As such, they are substrates for alkylation by electrophiles such as maleimide and iodoacetamide. [19] In cellular assays, N-ethylmaleimide (NEM) can inhibit retroviral infectivity in a concentration-dependent manner [20]. Modification of cysteine residues by alkylating agents such as iodoacetamide, 4-vinylpyridine and acrylamide has also been used in mass spectrometric peptide mapping for protein identification [21].

Although it is considered a noble metal, gold has a very rich chemistry. It is capable of forming coordination compound in both of its typical oxidation states (1+ and 3+). Gold(I) forms linear compounds, with coordination number 2, while gold(III) form square planar compounds with coordination number 4. Electron-rich metals such as gold are particularly suitable for designing metal-based zinc ejectors because, as soft Lewis acid electrophiles, they have high affinity for Cys residues. Without the proper tridimensional folding granted by Zn coordination, NCp7 is inactivated and unable to further recognize specific nucleic acid sequences. In this work, we make use of the Lewis acid electrophilic attack strategy, based on Au(I)-phosphine compounds, gold(III)(N^N) compounds and a gold(III)(C^N) organometallic compound.

Campinas, Brazil Raphael Enoque Ferraz de Paiva

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Abbreviations, Acronyms and Frequently Used Terms

"a_n" ion	Peptide fragment observed by MS/MS and CID. Corresponds to a decarbonylated "b_n" ion
apopeptide	Non-metallated protein
ATD	Arrival Time Distribution
"b_n" ion	Peptide fragment observed by MS/MS and CID. Originate from the nth amino acid from the N-terminus
bipy	2,2'-bipyridine
bnpy	2-benzylpyridine
CCDC	Cambridge Crystallographic Data Centre
CCS	Collision Cross-Section
CEM	T lymphoblast tumorigenic cell line, the associated disease is the acute lymphoblastic leukemia
CID	Collision Induced Dissociation
COSY	NMR experiment. Correlation Spectroscopy
CV	Cyclic Voltammetry
Cys	*L*-Cysteine
Cy_3P	Tricyclohexylphosphine
dien	Diethylenetriamine
DFT	Density Functional Theory calculations
dmap	4-dimethylaminopyridine
dmbipy	4,4'-dimethyl-2,2'-bipyridine
dmf	N,N-dimethylformamide
dmso	Dimethyl sulfoxide
Et_3P	Triethylphosphine
EXAFS	Extended X-Ray Absorption Fine Structure
F	Apo zinc finger protein. Appears along wiht the species that replaced Zn. One example of use is "AuF", indicating that a Au ion is coordinated to some of residues that were originally part of Zn coordination sphere in the protein
FP	Fluorescence Polarization

FWHM	Full width at half maximum
GAG	Core structural protein of HIV-1. After proteolytic processing, major viral proteins are obtained, such as the matrix (MA), capsid (CA) and nucleocapsid (NC)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, an organic zwitterionic compound used as buffering agent
HMBC	NMR experiment. Heteronuclear Multiple Bond Coherence
HSQC	NMR experiment. Heteronuclear Single-Quantum Coherence
HUVEC	Human Umbilical Vein Endothelial Cells
IMS	Ion Mobility Spectrometry
MS	Mass Spectrometry
N-Ac-Cys	*N*-Acetyl-*L*-Cysteine
NC	Nucleocapsid
NCp_7	Nucleocapsid protein 7 from HIV-1
Full-length	Full-length nucleocapsid protein from HIV-1, comprising both the ZnF1 and ZnF2 domains
ZnF_2	C-terminal ZnF domain of the nucleocapid protein from HIV-1
Zn_1F	Full-length nucleocapsid protein from HIV-1 containing Zn(II) in only one of its zinc finger domains
Zn_2F	Full-length nucleocapsid protein from HIV-1 containing Zn(II) in both zinc finger domains
NEM	N-ethylmaleimide
NMR	Nuclear Magnetic Resonance
PDB	Protein Data Bank
phen	1,10-phenantrholine
ROS	Reactive Oxygen Species
Sp1	Human transcription factor, comprises three ZnF domains
ZnF3	Third (C-terminal) ZnF domain of the human transcription factor protein Sp1
TD-DFT	Time-Dependent DFT
TWIM-MS	Traveling-wave Ion Mobility coupled with Mass Spectrometry
XANES	X-ray Absorption Near Edge Structure
XAS	X-ray Absorption Spectroscopy
"y_n" ion	Peptide fragment observed by MS/MS and CID. Originate from the nth amino acid from the C-terminus
ZnF	Zinc finger protein

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