Journal of Visualized Experiments

An in vitro Enzymatic Assay to Measure Transcription Inhibition by Gallium(III) and H3 5,10,15-tris(pentafluorophenyl)corroles

--Manuscript Draft--

Manuscript Number:	JoVE52355R3
Full Title:	An in vitro Enzymatic Assay to Measure Transcription Inhibition by Gallium(III) and H3 5,10,15-tris(pentafluorophenyl)corroles
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Corrole, RNA, transcription, inhibition, anticancer, DNA, binding, Actinomycin D, triptolide
Manuscript Classifications:	4.2: Organic Chemicals; 4.2.691: Organometallic Compounds; 4.3: Heterocyclic Compounds; 4.4: Polycyclic Compounds; 4.5: Macromolecular Substances; 95.51.6: biochemistry
Corresponding Author:	Melanie Pribisko California Institute of Technology Pasadena, CA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	melaniep@caltech.edu
Corresponding Author's Institution:	California Institute of Technology
Corresponding Author's Secondary Institution:	
First Author:	Grace Y. Tang
First Author Secondary Information:	
Other Authors:	Grace Y. Tang
	Ryan Kenneth Henning, B.S.
	Punnajit Lim
	John Termini, Ph.D.
	Harry B. Gray, Ph.D.
	Robert H. Grubbs, Ph.D.
Order of Authors Secondary Information:	
Abstract:	Chemotherapy often involves broad-spectrum cytotoxic agents with many side effects and limited targeting. Corroles are a class of tetrapyrrolic macrocycles that exhibit differential cytostatic and cytotoxic properties in specific cell lines, depending on the identities of the chelated metal and functional groups. The unique behavior of functionalized corroles towards specific cell lines introduces the possibility of targeted chemotherapy. Many anticancer drugs are evaluated by their ability to inhibit RNA transcription. Human cancer cells frequently become dependent on a single activated oncogene for survival; treatments blocking the expression of the oncogenes are effective in eliminating cancer cells. RNA transcription inhibition is therefore a useful way to identify potential anticancer drug candidates and learn more about their mechanism of action. Little is known about the molecular mechanism of action of corrole cytotoxicity. In this experiment, we consider two corrole compounds: gallium(III) 5,10,15-

	(tris)pentafluorophenylcorrole (Ga(tpfc)) and its freebase analogue 5,10,15-(tris)pentafluorophenylcorrole (tpfc). An RNA transcription assay was used to examine the inhibitive properties of the corroles. Five transcription reactions were prepared: DNA treated with Actinomycin D, triptolide, Ga(tpfc), tpfc at a [complex]:[template DNA base] ratio of 0.01, respectively, and an untreated control. The transcription reactions were analyzed after four hours using agarose gel electrophoresis and UV-Vis spectroscopy. There is clear inhibition by Ga(tpfc), Actinomycin D, and triptolide. This RNA transcription assay can be modified to provide more mechanistic detail by varying the concentrations of the anticancer complex, DNA, or polymerase enzyme, or by incubating the DNA or polymerase with the complexes prior to RNA transcription; these modifications would differentiate between an inhibition mechanism involving the DNA or the enzyme. Adding the complex after RNA transcription can be used to test whether the complexes degrade or hydrolyze the RNA. This assay can also be used to study additional anticancer candidates.
Author Comments:	CALIFORNIA INSTITUTE OF TECHNOLOGY Division of Chemistry and Chemical Engineering Dr. Melanie Pribisko. Ph.D. 1200 E. California Blvd. MC 127-72 E-mail: melaniep@caltech.edu October 13, 2014 JoVE Submission No.: 52355R2 Title: An in vitro Enzymatic Assay to Measure Transcription Inhibition by Gallium(III) and H3 5,10,15-tris(pentafluorophenyl)corroles Dr. Sephora Zaman, Ph.D. Science Editor, JoVE 1 Alewife Center Suite 200 Cambridge, MA 02140 Re: JoVE Submission No.: 52355R2 Dear Dr. Zaman, We thank the reviewers for thoroughly reading our manuscript and for their constructive comments. We have made several substantive changes to the manuscript in response to the reviewers' comments. In particular, we have emphasized that our manuscript provides an initial comparison of two standard transcription inhibitors compared to two cytotoxic corrole complexes, yet this experiment does not provide DNA binding information. We also clarified some of the language and incorporated more references, as requested by the reviewers. The details of the changes are described below: Reviewer Comments #1 & #2: The Introduction is very superficial with many incorrect and confusing statements and does not justify using Ga complex in the reported assay. For example line 117 contradicts line 118. Then line 104 contradicts line 106. (Reviewer #1) Insufficient references are provided for listed statements. For Introduction, reference 11 alone seems insufficient for cover the progress in the field of transcription inhibition by metal coordination compounds. For a more objective introduction, the later important work by Dunbar [Inorg. Chem. 2007, 46, 7494-7502] for Rh complexes, and some Cr complexes by Nair [Toxicology 2008, 251, 61-65] and Ru complexes by Ji [Inorg. Chem. 2009, 48, 5599-5601; J. Inorg. Biochem. 2010, 104, 576-582; Inorg. Chim. Acta 2011, 378, 140-147; Chem. Biodivers 2013, 10, 367-384] should be concluded. If the authors just want to mention the first work in this field, [Inorg. Chem. 2003, 42, 878-

884] should be used instead. Over the decade, the mechanism and structure-activity relationship have been thoroughly studied. These studies have become rather routine works. (Reviewer #2)

Response: Thank you very much for your suggestions. We have read through all of your suggested articles and have incorporated many into the manuscript. Corroles were used in this experiment to give a variety of transcription inhibitors, and also because Gallium complexes have been considered for anticancer therapeutics and have undergone clinical trials against hepatoma, lymphoma, bladder cancers, and other diseases, making them interesting to cancer researchers. After platinum, gallium is the second most promising metal to be considered for anticancer therapeutics and has also undergone many studies and investigations. Nitrate and chloride gallium salts have been evaluated in clinical trials against hepatoma, lymphoma, bladder cancers, and other diseases. Thank you very much for your comments; it is important that we justify the use of the Ga complex. This has been clarified in the manuscript and supported by the following references:

1.Chitambar, C.R., Narasimhan, J., Guy, J., Sem, D.S., O'Brien, W.J. Inhibition of ribonucleotide reductase by gallium in murine leukemic L1210 cells. Cancer Research. 51, 6199-6201 (1991).
2.Collery, P., Keppler, B., Madoulet, C., Desoize, B. Gallium in cancer treatment. Critical Reviews in Oncology / Hematology. 42 (3), 283-296, doi: 10.1016/S1040-8428(01)00225-6 (2002).
3.Chitambar, C.R. Medical applications and toxicities of gallium compounds. International Journal of Environmental Research and Public Health. 7 (5), 2337-2361, doi: 10.3390/ijerph7052337 (2010).
4.Chitambar, C.R. Gallium-containing anticancer compounds. Future Medicinal Chemistry. 4 (10), 1257-1272, doi: 10.4155/fmc.12.69 (2012).
5.Hedley, D.W., Tripp, E.H., Slowiaczek, P., Mann, G.J. Effect of gallium on DNA synthesis by human T-cell lymphoblasts. Cancer Research. 48 (11), 3014-8 (1988).
6.Seidman, A.D., Scher, H.I., Heinemann, M.H., Bajorin, D.F., Sternberg, C.N., Dershaw, D.D., Silverberg, M., Bosl, G.J. Continuous infusion gallium nitrate for patients with advanced refractory urothelial tract tumors. Cancer. 68 (12), 2561-5 (1991).

The manuscript as a whole has undergone changes in the language to improve clarity. For instance, regarding lines 104 and 106: functionalization of the scaffold does cause unique shifts in absorbance, which allows us to characterize the molecule, but its photophysical properties are not inherently affected. Lines 117 and 118 are referring to different cancer cell lines. We have adjusted the grammar to clarify this comparison.

Reviewer Comment #2: The knowledge on inhibition of DNA transcription in direct interaction with DNA may not have anything to do with effect of metal complexes on transcription. One may get the idea, based on this manuscript, that the methodology used readily translates to way of the action of metal complexes. Metals are redox active and thus many metal complexes work on level of redox-based metabolic pathways. Authors should indicate if the Ga complex studied gets into nucleus, any PK data available.

Response: Ga(III) is redox inactive, and therefore is not directly involved in redox-based metabolic pathways. Regardless, Ga(III) does exhibit cytotoxicity and has been investigated for therapeutic purposes. The purpose of this paper is to show that the RNA transcription inhibition assay is an affordable and versatile method to assess whether potential anticancer molecules merit further testing. The particular experiment in the paper was not meant to imply the mechanism of the inhibition; we suggested in the discussion section that there are many different ways which transcription inhibition could occur, and that additional experiments would be needed for full mechanistic understanding. This has been clarified in the manuscript, with emphasis on the following references:

1.Lim, P., Mahammed, A., Okun, Z., Saltsman, I., Gross, Z., Gray, H.B., Termini, J. Differential cytostatic and cytotoxic action of metallocorroles against human cancer cells: Potential platforms for anticancer drug development. Chemical Research in Toxicology. 25 (2), 400-409, doi: 10.1021/tx200452w (2012).
2.Richardson, D.R. Iron and gallium increase iron uptake from transferring by human

melanoma cells: Further examination of the ferric ammonium citrate-activated iron uptake process. Biochimica et Biophysica Acta. 1536 (1), 43-54, doi: 10.1016/S0925-4439(01)00034-5 (2001).

In the paper by Lim, et al., the studies show that each derivative of the metal corrole complex exhibits differential cytotoxicity towards human cells; for example sulfonated Ga corroles penetrate into the nucleus of the DU-145 cancer cell line, but do not do so for any of the other cancer cell lines tested in that study. In our paper, we focus on the general use of a transcription inhibition assay and not on nuclear penetration.

Reviewer Comment #3: The authors should pay more attention in the material and method section. For example, the recipe of TAE 50 X (section 3.2) isn't correct.

Response: The recipe for TAE 50x has been corrected to 2.0 M Tris-acetate and 0.05M EDTA. Thank you for pointing that out. We have reexamined each step in the materials and methods section and confirmed the accuracy.

Reviewer Comment #4: Line 181: Procedure of changing cis-[Pt(NH3)2Cl2] to cis-[Pt(NH3)2(H2O)2]2+ is not correct. As found in my own experience, this procedure is too easy to get completely converted hydrate. Then I changed to use the following procedure: "Activated cisplatin, cis-[Pt(NH3)2(OH2)2]2+, was prepared by reacting an aqueous solution of cisplatin with 2 equiv of AgNO3 for 1 day at room temperature in the dark. The reaction mixture was centrifuged for 10 min to remove AgCl. The aqueous layer was separated from the precipitate, and the solution was centrifuged for another 10 min; this process was repeated twice. [Inorg. Chem. 2004, 43, 1175-1183]" It should be noted that cisplatin must be handled avoiding light (this kind of notice can be found on the bottle of any cisplatin, solid or solution). If we stir it in water in light for several hours, as suggested here, only waste residue of cisplatin will be left, rather than activated one.

Response: First, we would like to clarify that in the original procedure, all platinum work was carried out in the dark. We regret not making that clear at the time. Second, we would like to thank the reviewer for providing experimental procedures from his/her own work. Third, we tested the procedure recommended by the reviewer, which differed from our original procedure only by the separation method (filtration vs. centrifugation).

When we redid the experiment using the explicit instructions for the preparation of cisplatin provided by Reviewer #2, we observed inconsistent results. As I mentioned in previous correspondence with the JoVE editor, we included two known transcription inhibitors in this study since the reviewers had concerns with the cisplatin. We decided to remove cisplatin as a known transcription inhibitor and only present data for the Actinomycin D and triptolide inhibitors. Ultimately, we came to this conclusion for three reasons: 1. of all the samples, only the cisplatin required advance preparation and purification. Setting the reaction up 24 hours in advance and using it within a small timeframe may not be convenient for users of the protocol. 2. the cisplatin reaction and purification needs to be carried out in the dark. Since I already work with light-sensitive compounds, my lab is set up for this work. In fact, my hood sash has a filter that blocks 99% of UV light. We cannot assume all labs and/or coworkers can tolerate working in the dark. 3. since the reaction is time and light sensitive, it is difficult to quantify the yield. We assume 100% conversion to active platinum, but that may not be the case and characterization is not easy. Since this is a protocol paper, we want it to be a resource with extremely reliable results and have taken the steps to do so.

Reviewer Comment #5: Line 191: "Note: ... Small amounts of DMSO (up to 1%) do not induce cytotoxicity in the cells (unpublished data)." Please be noted that this depiction is very dangerous. Dimethyl sulfoxide (1-10%) has been shown to accelerate strand renaturation and is believed to give the nucleic acid thermal stability against denaturation. That's why DMSO is used as a PCR cosolvent, helping improve yields, especially in long PCR. Therefore, "do not induce cytotoxicity" doesn't mean do not affect DNA transcription. If DMSO affects transcription but not tumor cell activity, the conclusion might be "inhibition of DNA transcription is not relative to antitumor activity".

Response: In the manuscript, "up to 1%" meant "less than 1%". We acknowledge the

	importance of using less than 1% DMSO and have edited the text to more precise language. Reviewer Comment #6: Line 225: Lithium chloride precipitation. This part is somewhat not necessary because DNA and DNA polymerase do not really affect the result of RNA quantification by gel electrophoresis. If one really wants to quantify RNA by UV, a RNA spin column could get over 90 % RNA from mixture of DNA and protein within 2 minutes, while precipitation method may give apparently lower recovery and purity and is time cost. Response: We believe it is important to purify the transcription reaction for two reasons: 1. it stops the transcription reaction, preventing error being introduced due to different reaction time lengths, and preventing any degradation of the RNA product from the enzymes in the solution. and 2. we found gels run after a purification step to be much clearer than gels run with all the reaction components. We redid the experiment using mini Quick Spin RNA Columns to purify the RNA, and we have included that change in our updated procedure. Reviewer Comments #7 and #8: Figure legends have insufficient information, for example in Figures 2 and 3 no information about concentration of Ga complex is given (Reviewer #1). Also, it should be considered to improve the figure legends, especially the one of figure 3, that doesn't explain well the differences between sample 1-4 and 5-8 (Reviewer #3). Response: All figure legends and captions have been edited for maximum clarity. In Figure 2, the concentration is reflected in the x-axis labeled "Treatments", and is measured on a logarithmic scale in moles. In Figure 3, the ratio of [metal complex] to [DNA] was 0.01. Since there was 0.43 pmol DNA, all inhibitors are at 4.3 fmol concentration. Reviewer Comment #7: Figure 3. Please show the whole picture of gel with ladder (size marker). Response: Figure 3 has been changed to include the DNA ladder, and modified to improve clarity. Reviewer Comment #8: Molar absorptivities are needed for uv/vis spectra. Response: We have corrected this oversight. We hope that these changes to the manuscript satisfy both the reviewers and the editorial board at JoVE. We wish to publish an article of high quality that will be of interest to researchers in the biological and chemical sciences, therefore, please let us know if there is anything else we can do to improve the content of our article. Sincerely, Melanie Pribisko
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

CALIFORNIA INSTITUTE OF TECHNOLOGY

Division of Chemistry and Chemical Engineering
Mail Code 127-72
Pasadena, California 91125

Melanie A. Pribisko
Senior Postdoctoral Scholar
(626) 395-6050
E-mail: melaniep@caltech.edu

May 29, 2014

Dr. Avital Braiman
Director of Editorial
Journal of Visualized Experiments
1 Alewife Center
Suite 200
Cambridge MA 02140

Dear Dr. Avital Braiman,

Please find attached a manuscript entitled "An *in vitro* Enzymatic Assay to Measure Transcription Inhibition by Gallium (III) and H_3 5,10,15-tris(pentafluorophenyl)corroles", which we would like to have considered for publication in the *Journal of Visualized Experiments.* In addition, we have also submitted "Rotary Evaporation to Remove Solvent", in partial fulfilment of the requirement to have the publication fees waived for our JoVE video-article.

Our manuscript describes the protocol of a mRNA transcription assay and the results from the incorporation of anti-cancer complexes into the assay to show transcription inhibition. Corrole toxicity to cancer cells is well-established, however, little is known about the molecular nature or mechanism of action of corrole cytotoxicity. In our experiment, we examine the affect of cisplatin and two corrole compounds, gallium (III) 5,10,15-(tris)pentafluorophenylcorrole (Ga(tpfc)) and its freebase analogue 5,10,15-(tris)pentafluorophenylcorrole (tpfc), on transcription using a commercially available assay from Life Technologies. The transcription reactions were analyzed at one and three hours using agarose gel electrophoresis and UV-Vis spectroscopy. There is clear inhibition by both of the corroles at the one and three hour time points. UV-Vis spectroscopy was used to quantify levels of RNA production; our results show Ga(tpfc) and tpfc exhibit greater inhibition than did *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ at all time points.

As this RNA transcription assay is highly adaptable, we feel it is of great value to the JoVE audience. We provided detailed instructions and reagent information in order for viewers to be able to perform their own assays using either the commercially available kit or through individual preparation of the reagents. The assay can provide detailed information of transcription inhibition by exogenous molecules of interest, as demonstrated in this manuscript, or for many other general purposes that are of biological interest.

Since our work bridges synthetic corrole chemistry and biochemical processes, we suggest reviewers familiar with metalloproteins, corroles, and transcription. Immediately following this letter is a list of experts in the field who we believe would serve as excellent referees for this manuscript.

Sincerely,

Melanie Pribisko

Suggested peer reviewers:

Dr. Karn Sorasanee
Children's Hospital of Los Angeles
ksorasanee@chla.usc.edu

Dr. Stephen O'Shea
Roger Williams University
soshea@rwu.edu

Dr. Lali K. Medina-Kauwe
Cedars-Sinai
lali.medinakauwe@cshs.org

Dr. Donald Walker
The Aerospace Corporation
don.walker@aero.org

Mr. Tim Johnstone
Massachusetts Institute of Technology
timothyj@mit.edu

Dr. Yves Pommier
National Cancer Institute
pommier@nih.gov

TITLE:
An *in vitro* Enzymatic Assay to Measure Transcription Inhibition by Gallium(III) and H_3 5,10,15-tris(pentafluorophenyl)corroles

AUTHORS:

Tang, Grace Y.
Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, California 91125, United Sates
gytang@caltech.edu

Pribisko, Melanie A.
Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, California 91125, United Sates
melaniep@caltech.edu

Henning, Ryan K.
Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, California 91125, United Sates
rhenning@caltech.edu

Lim, Punnajit
Department of Molecular Medicine
Beckman Research Institute of the City of Hope
Duarte, California 91010, United States
plim@coh.org

Termini, John
Department of Molecular Medicine
Beckman Research Institute of the City of Hope
Duarte, California 91010, United States
jtermini@coh.org

Gray, Harry B.
Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, California 91125, United Sates
hbgray@caltech.edu

Grubbs, Robert H.
Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, California 91125, United Sates

rhg@caltech.edu

CORRESPONDING AUTHOR:
Robert H. Grubbs
rhg@caltech.edu
626-395-6003

KEYWORDS:
Corrole, RNA, transcription, inhibition, anticancer, DNA, binding, Actinomycin D, triptolide

SHORT ABSTRACT:
Gallium(III) 5,10,15-(tris)pentafluorophenylcorrole and its freebase analogue exhibit low micromolar cell cytotoxicity. This manuscript describes an RNA transcription reaction, imaging RNA with an ethidium bromide-stained gel, and quantifying RNA with UV-Vis spectroscopy, in order to assess transcription inhibition by corroles and demonstrates a straightforward method of evaluating anticancer candidate properties.

LONG ABSTRACT:

Chemotherapy often involves broad-spectrum cytotoxic agents with many side effects and limited targeting. Corroles are a class of tetrapyrrolic macrocycles that exhibit differential cytostatic and cytotoxic properties in specific cell lines, depending on the identities of the chelated metal and functional groups. The unique behavior of functionalized corroles towards specific cell lines introduces the possibility of targeted chemotherapy.

Many anticancer drugs are evaluated by their ability to inhibit RNA transcription. Here we present a step-by-step protocol for RNA transcription in the presence of known and potential inhibitors. The evaluation of the RNA products of the transcription reaction by gel electrophoresis and UV-Vis spectroscopy provides information on inhibitive properties of potential anticancer drug candidates and, with modifications to the assay, more about their mechanism of action.

Little is known about the molecular mechanism of action of corrole cytotoxicity. In this experiment, we consider two corrole compounds: gallium(III) 5,10,15-(tris)pentafluorophenylcorrole (Ga(tpfc)) and freebase analogue 5,10,15-(tris)pentafluorophenylcorrole (tpfc). An RNA transcription assay was used to examine the inhibitive properties of the corroles. Five transcription reactions were prepared: DNA treated with Actinomycin D, triptolide, Ga(tpfc), tpfc at a [complex]:[template DNA base] ratio of 0.01, respectively, and an untreated control.

The transcription reactions were analyzed after four hours using agarose gel electrophoresis and UV-Vis spectroscopy. There is clear inhibition by Ga(tpfc), Actinomycin D, and triptolide.

This RNA transcription assay can be modified to provide more mechanistic detail by varying the concentrations of the anticancer complex, DNA, or polymerase enzyme, or by incubating the DNA or polymerase with the complexes prior to RNA transcription; these modifications would differentiate between an inhibition mechanism involving the DNA or the enzyme. Adding the complex after RNA transcription can be used to test whether the complexes degrade or hydrolyze the RNA. This assay can also be used to study additional anticancer candidates.

INTRODUCTION:

Chemotherapy often involves broad-spectrum cytotoxic agents with undesired side effects and limited targeting, yet with greater understanding of cancer biology, there is an ever increasing demand for anticancer agents with higher cancer-targeting efficacy and fewer side effects.[1] Human cancer cells frequently become dependent on a single activated or overexpressed oncogene for survival.[2] Thus, many anticancer drugs are evaluated by their ability to inhibit RNA transcription. Treatments that block the expression of these transforming genes are effective in eliminating cancer cells and lead to cell death.[3] Transformed cells are more sensitive to disruptions in RNA transcription than are corresponding normal cells.[4] Anticancer drugs that inhibit transcription are expected to selectively inhibit the expression of the oncogenes which are necessary for the cancer cell to survive.[5] Consequently, RNA transcription inhibition is a useful way to identify potential anticancer drug candidates and learn more about their mechanism of action. This protocol demonstrates that Ga(tpfc) inhibits RNA transcription on the same order as the chemotherapy drugs Actinomycin D and triptolide; similar comparisons can be made using this protocol with other anticancer drug candidates. Actinomycin D is a RNA transcription inhibitor commonly used to treat gestational trophoblastic cancer, testicular cancer, Wilm's tumor, rhabdomyosacoma, and Ewing's sarcoma[6]. Actinomycin D has been used in cancer therapy for nearly fifty years since it was first approved by the FDA in 1964.Triptolide is a selective transcription inhibitor that has been investigated *in vitro* and in various tumor-bearing animal models for 30 years.[7]

The amphiphilic macrocyclic nature of corroles imparts significant advantages over other drug classes such as small molecules or biologics.[8-14] The macrocyclic character allows for cellular permeability that is greater than expected for such large molecules, and they are large enough to interact with macromolecular surfaces, such as those of proteins.[8] Corroles are known to form tight noncovalent complexes with biomolecules and drugs.[10] In addition to the inherent cytotoxicity of the corrole framework, we have demonstrated that a sulfonated corrole acts as a carrier molecule for chemotherapeutic agents, specifically the DNA-intercalating anthracycline drug doxorubicin. When the sulfonated corrole was coadministered with doxorubicin, a 3-fold enhancement in the IC_{50} of doxorubicin was observed for DU-145 cells.[9] The corrole framework is stable and has inherent absorbance and fluorescence properties that, when functionalized, undergo unique absorbance shifts that can be used for characterization.[10] Functionalization of the scaffold does not inherently affect the photophysical properties of the corrole,[9-15] but, as seen with a sulfonated corrole, selectively modifying the

framework of the corrole can substantially change its biological properties.[16] We previously evaluated six metallocorroles against seven human cancer cell lines. The results indicate that toxicity toward human cancer cells is dependent on the specific metal ion, as well as functional group substitution. For instance, sulfonated gallium corroles experienced high cellular uptake and penetrated selectively into the nucleus of brain metastatic prostate cancer cells (DU-145); the same corrole, though it does not penetrate into the nucleus of other cell lines, exhibits greater cytotoxicity for breast (MDA-MB-231), melanoma (SK-MEL-28), and ovarian (OVCAR-3) cancer cells than for prostate cancer.[9]

Initial cell-based assays indicate that these compounds show promise as anti-cancer therapeutic agents, which merits further investigation into the mechanism of action. Transcription inhibition is observed with certain organometallic complexes[17-27] and we sought to examine this process as a possible mechanism for the cytotoxic behavior of the corrole family. This transcription assay provides a straightforward, inexpensive, and facile method for assessing transcription inhibition, which will lead to more detailed information about the effects of these molecules in live cells.

Here, the transcription inhibition of gallium(III) 5,10,15-(tris)pentafluorophenylcorrole (Ga(tpfc)) and its freebase analogue 5,10,15-(tris)pentafluorophenylcorrole (tpfc) (**Figure 1**) are tested. Unlike some transition metal complexes, gallium(III) is redox inactive and therefore is not directly involved in the redox process of redox-based metabolic pathways.[28] Regardless, gallium(III) does exhibit cytotoxic properties and has been investigated for therapeutic purposes. Gallium is the second most promising metal for anticancer therapeutics after platinum and has undergone many studies and investigations; nitrate and chloride gallium salts have been evaluated in clinical trials against hepatoma, lymphoma, bladder cancers, and other diseases.[29-34] Gallium(III) is therefore ideal for anticancer corrole studies. Initial data show Ga(tpfc) and tpfc have low GI_{50}, the drug concentration necessary to inhibit 50% of maximal cell proliferation, with various cancer cell lines (see **Figure 2**); this affirms the validity of further experiments on these two compounds to determine their inhibitive properties. We compare these compounds with the common anticancer drugs Actinomycin D and triptolide. Actinomycin D intercalates DNA, inhibits RNA elongation, and induces apoptosis in certain cell line at picomolar concentrations.[6,35-37] Triptolide has shown to inhibit tumor growth; it binds to human XPB/ERCC3, a subunit of transcription factor TFIIH, leading to inhibition of RNA polymerase II activity.[6-7,38-40]

While it is commonly known that corroles exhibit cytotoxic properties, there exists little information about the different mechanisms arising from functionalization. Corrole inhibition of RNA transcription would offer greater insight on their interactions with biomacromolecules. Other complexes known to bind to DNA, such as dirhodium(II,II) complexes, chromium(III) complexes, ruthenium(II) polypyridyl complexes, rhodium(III) complexes, and various others, were subjected to RNA transcription assays,[18-27] resulting in greater understanding of their interactions with biomacromolecules. This facile and widely available experiment is also a good initial test to assess the cytotoxicity properties of a given molecule and determine whether it merits further

biological testing. The RNA transcription assay also allows for many modifications, such as varying the quantity of compound or enzymes used; varying the incubation period, reaction time and sample time points; and varying the DNA template length and sequence, among other variables of interest, thus potentially providing a large amount of data. This transcription assay is also readily available as affordable kits with all necessary reaction components provided, although components can be bought and prepared individually. In these experiments, we use a commercially available kit known to have high yield. [41]

To assess transcription inhibition, we use two methods: agarose gel electrophoresis and UV-Vis spectroscopy. Agarose gel electrophoresis is a simple and effective method for separating, identifying, and purifying 0.5- to 25-kb DNA and RNA fragments.[42] UV-Vis spectroscopy can be used to determine the concentration and purity of RNA.[43]

PROTOCOL:

Note: When working with RNA maintain a clean working environment to avoid contamination by DNase and RNase enzymes that degrade DNA and RNA. Ensure that pipette tips and tubes are DNase and RNase free. It is also helpful to wipe down lab surfaces and equipment such as pipettes, tube holders, etc. with a decontamination solution.

1. RNA transcription with corrole treatment

1.1) Prepare the corrole and inhibitor compounds in a 0.01:1 molar ratio of [complex]:[DNA].

Note: In our case, the ratio was 4.3 fmol complex: 0.43 pmol DNA, where the DNA template used was a pET-28c vector with ligA gene from *E. coli* under control of the T7 promoter. Other DNA with the T7 promoter are also valid candidates for running the transcription assay.

1.1.1) Dissolve Actinomycin D, triptolide, tpfc, and Ga(tpfc) in dimethyl sulfoxide (DMSO) in clean, separate containers. Obtain the final concentration of a 0.01:1 molar ratio of [complex]:[DNA] by using serial dilutions with nuclease-free water.

1.1.1.1) Dissolve 1 mg Actinomycin D in 1.8 mL DMSO. Aliquot 10 µL to a separate container and dilute to 1 mL with nuclease-free water to create a 1/100 dilution. Aliquot 1 µL of the dilution to a separate container and dilute to 1 mL with nuclease-free water to create a 1/1000 dilution. Note: The concentration of the final solution of Actinomycin D is 4.3 fmol.

1.1.1.2) Dissolve 1 mg triptolide in 0.64 mL DMSO. Aliquot 1 µL to a separate container and dilute to 1 mL with nuclease-free water to create a 1/1000 dilution. Aliquot 1 µL of the dilution to a separate container and dilute to 1 mL with nuclease-free water to create a second 1/1000 dilution. Note: The concentration of the final solution of triptolide is 4.3

fmol.

1.1.1.3) Dissolve 1 mg tpfc in 2.9 mL DMSO. Aliquot 10 µL to a separate container and dilute to 1 mL with nuclease-free water to create a 1/100 dilution. Aliquot 1 µL of the dilution to a separate container and dilute to 1 mL with nuclease-free water to create a 1/1000 dilution. Note: The concentration of the final solution of tpfc is 4.3 fmol.

1.1.1.4) Dissolve 1 mg Ga(tpfc) in 2.6 mL DMSO. Aliquot 10 µL to a separate container and dilute to 1 mL with nuclease-free water to create a 1/100 dilution. Aliquot 1 µL of the dilution to a separate container and dilute to 1 mL with nuclease-free water to create a 1/1000 dilution. Note: The concentration of the final solution of Ga(tpfc) is 4.3 fmol.

Note: These corroles and inhibitors are not soluble in water and must be pre-dissolved in DMSO. Small amounts of DMSO (below 1%) do not induce cytotoxicity in the cells (unpublished data).

1.2) Prior to beginning the transcription reaction, prepare the necessary reagents individually or purchase them from a commercial vendor.

1.2.1) Thaw on ice all frozen reagents (refer to Table 1 for a list of frozen reagents).

1.2.2) Allow the 10X Reaction Buffer and the 4 ribonucleotide (ATP, CTP, GTP, and UTP) solutions time to mix until they are completely dissolved in solution.

1.2.3) Briefly centrifuge all reagents to prevent loss of material on the rim of the tube. Once thawed, store ribonucleotides on ice while keeping the 10X Reaction Buffer at room temperature.

1.2.4) Combine reagents for transcription reaction at room temperature (refer to Table 2 for a list of reagents).

Note: The spermidine in the 10x Reaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice rather than at room temperature.

1.2.5) Mix thoroughly by flicking the tube or pipetting the mixture up and down gently. After mixing, centrifuge briefly to collect reaction mixture at the bottom of the tube.

1.2.6) Incubate the reaction mixture at 37 °C for a total of 2-4 hr.

Note: The necessary reaction times will vary with DNA template size and sequence. This step may require optimization for specific sequences. Shorter templates may require longer reaction times for a high yield of total RNA.

1.2.7) Remove 4 µL aliquots from each reaction after each hour and store at -20 °C. Modify as needed for desired timepoints.

2. RNA spin column

2.1) Following the transcription reaction, purify the RNA using microcentrifuge-compatible chromatography columns. Note: Use of RNA spin columns to purify RNA with greater than 20 bases results in greater than 80% recovery.[44]

Note: Column chromatography is a common and convenient method to purify RNA. Other purification methods also exist such as Lithium Chloride precipitation, phenol:chloroform extraction, isopropanol precipitation, as well as other commercially available kits.

2.1.1) Evenly resuspend the Sephadex matrix in the column buffer by vigorously inverting the column three or more times. Do this by flicking the column sharply.

2.1.2) Remove the top cap from the column. There will be some residual Sephadex matrix in the cap; if the cap is filled with the matrix, replace the cap onto the column and repeat the previous step until the majority of the matrix is in the column.

2.1.3) Snap off the bottom tip of the column. Note: Some liquid may escape from the column.

2.1.4) Pack the column.

2.1.4.1) Place column in a sterile (RNase and DNase free) 1.5 mL microcentrifuge tube.

2.1.4.2) Centrifuge the tube in a tabletop centrifuge at 1,000 x g for 1 min.

2.1.5) Discard the 1.5 mL microcentrifuge tube with eluted buffer from the column.

2.1.6) Immediately place the column in a new sterile 1.5 mL microcentrifuge tube and pipette the sample to the center of the column bed. Use 20-50 µL of sample to avoid overloading the column.

2.1.7) Centrifuge the tube in a tabletop centrifuge at 1,000 x g for 1 min. Note: The eluent is the purified RNA sample.

3. Agarose Gel Electrophoresis (1% agarose gel) [42]

Note: Ethidium bromide fluoresces upon binding to DNA and RNA, therefore these biomolecules can be visualized with UV light by incubating them with 0.5 µg/mL ethidium bromide solution.

3.1) Prepare a 1000x stock solution of ethidium bromide (0.5 mg/mL) by dissolving 5 mg ethidium bromide in 10 mL of water.

3.2) Prepare Tris Acetate EDTA (TAE) running buffer for gel electrophoresis. To make a

50x TAE buffer combine 2.0 M Tris-acetate with 0.05 M Ethylenediaminetetraacetic acid (EDTA) and adjust the pH to 8.5.

3.3) Prepare a 1% (weight/volume) agarose gel by dissolving 10 g UltraPure Agarose in 1 L 1x TAE buffer and melting the agarose with a conventional microwave oven. Once it has cooled to 50°C, add 1 mL of 1000x ethidium bromide to the 1% agarose gel solution, mix by gently swirling or by inverting in a closed container, then pour the agarose solution into a gel-casting platform and allow the gel to solidify at room temperature.

CAUTION: Ethidium bromide is a mutagen and potential carcinogen. Wear gloves and handle solutions carefully. For proper handling procedures of ethidium bromide, please see: http://www.sciencelab.com/msds.php?msdsId=9927667.

3.4) After the gel has solidified, place the set gel in the electrophoresis tank. Add enough TAE buffer to cover the gel until the wells are submerged. Check that the level of the TAE buffer is approximately 1 mm above the level of the gel. Remove the gel comb.

Note: Removing the comb after wells are submerged prevents air pockets from being trapped in the wells.

3.5) Prepare the RNA samples. Combine 1 µL of each purified sample aliquot with 1 µL Gel Loading Buffer (or with 1 µL 10x loading buffer and diluted to 10 µL with nuclease-free water) and mix thoroughly by pipetting up and down with a micropipette.

Note: 10x loading buffer contains 20% Ficoll 400, 0.1 M Na_2EDTA, pH 8.0, 1.0% SDS, 0.25% bromophenol blue. 0.25% xylene cyanol may also be added to the loading buffer, as it runs ~50% as fast as bromophenol blue and can be useful for monitoring very long runs.[42]

3.6) Load the gel with each sample by carefully pipetting the solution into the bottom of each well. Take care not to leave any air bubbles or mix samples between wells.

Note: A RNA ladder can also be used to determine the size of the transcript.

3.7) Attach the leads so that the DNA will migrate into the gel towards the positive lead. Set the voltage to the desired level, typically 1 to 10 V/cm of gel. Run the gel for however long is sufficient for significant separation between the DNA and RNA, using the migration of dyes to monitor the progress of the separation.

Note: A 23 cm x 25 cm gel at 250 V will take approximately one hour, while an 8 cm x 8 cm gel at 150 V will take approximately 20 minutes. Smaller RNA fragments have better resolution when run at higher voltages.

3.8) Turn off the power supply when the dye from the loading buffer has migrated a

distance judged sufficient for separation between the DNA and RNA.

3.9) Image the RNA under UV light as the ethidium bromide will fluoresce.

3.10) Take a picture of the image and compare fluorescence intensities of the purified RNA from each condition.

4. RNA quantification via UV-Vis spectroscopy

4.1) Place 2 µL H_2O on a NanoDrop 2000, or similar machine, and measure the Blank.

4.2) Next, place 2 µL of each RNA sample, following purification, on the spectrophotometer and measure UV-Vis from a wavelength range of 200 nm to 800 nm. Note: An A_{260} reading of 1.0 is equivalent to about 40 µg/ml of RNA, and pure RNA has an A_{260}/A_{280} ratio of 2.1.[45]

4.3) In the case that absorbance is above 1 O.D., dilute the samples in nuclease-free water. Start with a 1:4 dilution if the original concentration is too high.

4.4) Use graphing software to plot the various samples and compare O.D. at 260 nm.

REPRESENTATIVE RESULTS:

RNA Transcription Qualitatively Assessed by Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to image the transcribed RNA. Ethidium bromide fluoresces upon binding (λ_{em} = 605 nm, λ_{ex} = 210 nm, 285 nm)[46] allowing imaging of RNA. Darker bands in the gel correspond to higher concentrations of RNA. If Actinomycin D, triptolide, or either corrole complex inhibits RNA transcription, the production of RNA is reduced and the band will appear lighter. Using this concept, relative inhibition can be assessed.

Figure 3 shows the ethidium bromide stained agarose gel (1%) of RNA transcription reactions pre-treated with no complex, Actinomycin D, triptolide, tpfc, or Ga(tpfc) at a [complex]:[template DNA base] ratio of 0.01:1. Actinomycin D and triptolide show a clear decrease of RNA compared to that of the control, as expected of these long-studied inhibitors. The Ga(tpfc) band also has a very low level of RNA, while the tpfc band shows little to no inhibition and exhibits the same relative intensity as the control.

RNA Transcription Quantified by UV-Vis Spectroscopy

UV-Vis measurements were taken for each sample after undergoing RNA transcription for 4 hours and purified with RNA spin column chromatography. The spectra of wavelengths 220 nm to 350 nm are shown in **Figure 4**, with the λ_{max} occurring at 260 nm, corresponding to RNA absorption, and an extinction coefficient of 0.025 $(\mu g/ml)^{-1}$ cm^{-1}. The absorbance at 260 nm and 280 nm are reported in Table 3. An A_{260} reading

of 1.0 is equivalent to about 40 µg/ml of RNA, and pure RNA has an A_{260}/A_{280} ratio of 2.1, allowing for quantitative calculations of RNA produced during the transcription reaction and an assessment of the purity of the RNA after using the RNA spin column.[44] Three of the four inhibitor-treated transcription reactions yielded less RNA than the control, with Ga(tpfc)-treated DNA transcribing only 0.07 times as much RNA as the untreated DNA. tpfc-treated DNA showed no apparent inhibition. All samples have an A_{260}/A_{280} ratio of approximately 2.2, indicating relatively pure samples. Purification by RNA spin columns removes short RNA strands 20 or fewer nucleotides long. Residual DNA or strands longer than 20 nucleotides may be present in what is considered the purified samples. These slight impurities would result in an A_{260}/A_{280} ratio slightly greater than 2.1.

Figure Legends:

[place figure 1 here]

Figure 1. Molecular structures of Gallium(III) 5,10,15-(tris)pentafluorophenylcorrole (Ga(tpfc)) and the freebase analogue 5,10,15-(tris)pentafluorophenylcorrole (tpfc).

[place figure 2 here]

Figure 2. Cytotoxicity curves and GI_{50} values of corroles in prostate (DU-145) (gray), breast (MDA-MB-231) (blue), skin (SK-MEL-28) (orange), and ovarian (OVCAR-3) (green) cancer cell lines. Cells were incubated with **(A)** Ga(tpfc) and **(B)** tpfc, followed by determination of viability using the MTS-based colorimetric cell viability assay according to the manufacturer's protocol.

[place figure 3 here]

Figure 3. Ethidium bromide stained agarose gel (1%) of transcription reactions after 4 hours of incubation. Lane 1 is a 1000 bp DNA ladder as a standard. Lanes 2-6 show the RNA transcribed from 0.43 pmol DNA and treated with 4.3 fmol of each inhibitor (a [complex]:[template DNA base] ratio of 0.01): the control (lane 2), Actinomycin D (lane 3), triptolide (lane 4), tpfc (lane 5), and Ga(tpfc) (lane 6).

[place figure 4 here]

Figure 4. UV-Vis spectrum of 1:200 dilution of transcribed RNA at after 4 hours of incubation. The DNA template for the transcription reaction was treated with no complex, or with Actinomycin D, triptolide, tpfc, or Ga(tpfc) at a [complex]:[template DNA base] ratio of 0.01:1. The RNA concentration is measured by O.D. at 260 nm.

Table 1. List of Frozen Reagents.

Table 2. List of Reagents for Transcription Reaction.

Table 3. Concentration and purity of transcribed RNA after 4 hours measured by UV-Vis Spectroscopy. The extinction coefficient of single-stranded RNA is 0.025 $(\mu g/ml)^{-1}$ cm^{-1}, an A_{260} reading of 1.0 is equivalent to about 40 µg/ml of RNA, and pure RNA has an A_{260}/A_{280} ratio of 2.1.[45]

[place table 3 here]

DISCUSSION:

This assay demonstrates that the addition of Ga(tpfc) inhibits RNA transcription comparably to the known DNA-binding anticancer complexes Actinomycin D and triptolide. The cytotoxic behavior of Ga(tpfc) (GI_{50} = 58.1-154.7 µM) may due to its inhibitive properties. Since no transcription inhibition was observed in tpfc, the cytotoxicity of tpfc is not due to RNA transcription inhibition but is caused by other means not yet studied.

In the four transcription reactions performed, DNA was treated with Actinomycin D, triptolide, tpfc, or Ga(tpfc), at a [complex]:[template DNA base] ratio of 0.01, respectively, or an untreated control. The transcription reaction reagents were combined and the transcription reaction was allowed to proceed for 4 hours at 37 °C. The transcribed RNA was purified through a RNA spin column and the yield analyzed by UV-Vis and gel electrophoresis. The nature of transcription inhibition can be further investigated using this same technique with various modifications, or the compounds can be subjected to alternative *in vitro* and *in vivo* studies. Additional experiments which may help determine the molecular nature of the inhibition include X-ray crystallography of possible corrole-DNA adducts or computational modeling of the transcription reaction. The objective of this experiment was to determine whether the compounds inhibit transcription in order to collect information on their potential anticancer properties. That objective was achieved: tpfc exhibited no inhibition, while Ga(tpfc) exhibited clear inhibition and merits further study.

The RNA transcription assay can be modified to provide more mechanistic detail. Addition of the anticancer agents (e.g., the corrole complexes) after the transcription reaction is complete can show whether the complexes degrade or hydrolyze the RNA. Another recommended experiment is to conduct the transcription reaction with all components with the exception of the RNA polymerase enzyme 10-fold lower to allow for differentiation between an inhibition mechanism involving the DNA or the enzyme. Finally, incubation of the DNA or the polymerase with the complexes prior to RNA transcription would allow for increased binding between the complex and the respective target, ascertaining whether the complex inhibitive qualities are involved in DNA or polymerase binding, respectively.

ACKNOWLEDGMENTS:

We sincerely thank Dr. Cindy N. Chiu for help with gel electrophoresis, and Andy Zhou and Michael Grodick for their generous donation of DNA and restriction enzyme. We gratefully acknowledge Professor J. Heath and Professor D. Prober for generous access to equipment and materials. We thank Dr. Karn Sorasaenee for helpful suggestions. Funding was provided by Johnson & Johnson and USC Y86786.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES

1. American Cancer Society [Internet]. Atlanta: American Cancer Society; c2014 [cited 2014 April 30]. Available from: http://www.cancer.org.

2. Weinstein, I.B. Addiction to oncogenes—The Achilles heel of cancer. *Science*. **297**, 63-64 (2002).

3. Derheimer, F.A., Chang, C.W., Ljungman, M. Transcription inhibition: A potential strategy for cancer therapeutics. *Eur. J. Cancer*. **41** (16), 2569-2576, doi: 10.1016/j.ejca.2005.08.012 (2005).

4. Koumenis, C., Giaccia, A. Transformed cells require continuous activity of RNA polymerase II to resist oncogene-induced apoptosis. *Mol. Cell. Biol.* **17** (12), 7306-16 (1997).

5. Stellrecht, C.M., Chen, L.S. Transcription Inhibition as a Therapeutic Target for Cancer. *Cancers*. **3** (4), 4170-4190, doi:10.3390/cancers3044170 (2011).

6. Bensaude, O. Inhibiting eukaryotic transcription: Which compound to choose? How to evaluate its activity? *Transcription*. **2** (3), 103-108, doi: 10.4161/trns.2.3.16172 (2011).

7. Liu, Q. Triptolide and its expanding multiple pharmacological functions. *International Immunopharmacology*. **11** (3), 377-83, doi: 10.1016/j.intimp.2011.01.012 (2011).

8. Mahammed, A., Gray, H.B., Weaver, J.J., Sorasaenee, K., Gross, Z. Amphiphilic corroles bind tightly to human serum albumin. *Bioconjugate Chemistry.* **15** (4), 738-746, doi: 10.1021/bc034179p (2004).

9. Lim, P., *et al*. Differential cytostatic and cytotoxic action of metallocorroles against human cancer cells: Potential platforms for anticancer drug development. *Chemical Research in Toxicology*. **25** (2), 400-409, doi: 10.1021/tx200452w (2012).

10. Bendix, J., Dmochowski, I.J., Gray, H.B., Mahammed, A., Simkhovich, L., Gross, Z. Structural, electrochemical, and photophysical properties of gallium(III)

5,10,15-tris(pentafluorophenyl)corrole. *Angewandte Chemie-International Edition.* **39** (22), 4048-4051, doi: 10.1002/1521-3773(20001117)39:22<4048::AID-ANIE4048>3.0.CO;2-7 (2000).

11. Hwang, J.Y., Gross, Z., Gray, H.B., Medina-Kauwe, L.K., Farkas, D.L. Ratiometric spectral imaging for fast tumor detection and chemotherapy monitoring in vivo. *Journal of Biomedical Optics.* **16** (6), 1-6, doi: 10.1117/1.3589299 (2011).

12. Agadjanian, H., *et al*. Tumor detection and elimination by a targeted gallium corrole. *Proceedings of the National Academy of Sciences of the United States of America.* **106** (15), 6105-6110, doi: 10.1073/pnas.0901531106 (2009).

13. Hwang, *et al*. Photoexcitation of tumor-targeted corroles induces singlet oxygen-mediated augmentation of cytotoxicity. *Journal of Controlled Release.* **163** (3), 368-373, doi: 10.1016/j.jconrel.2012.09.015 (2012).

14. Hwang, J.Y., *et al*. Investigating photoexcitation-induced mitochondrial damage by chemotherapeutic corroles using multimode optical imaging. *Journal of Biomedical Optics.* **17** (1), 11, doi: 10.1117/1.JBO.17.1.015003 (2012).

15. Hwang, J.Y., *et al*. A Mechanistic Study of Tumor-Targeted Corrole Toxicity. *Molecular Pharmaceutics.* **8** (6), 2233-2243, doi: 10.1021/mp200094w (2011).

16. Saltsman, I., Mahammed, A., Goldberg, I., Tkachenko, E., Botoshansky, M., Gross, Z. Selective substitution of corroles: Nitration, hydroformylation, and chlorosulfonation. *Journal of the American Chemical Society.* **124** (25), 7411-7420, doi: 10.1021/ja025851g (2002).

17. Gershman, Z., Goldberg, I., Gross, Z. DNA Binding and Catalytic Properties of Positively Charged Corroles. *Angewandte Chemie*. **46** (23), 4320-4324, doi: 10.1002/anie.200700757 (2007).

18. Fu, P.K., Bradley, P.M., Turro, C. Stabilization of duplex DNA structure and suppression of transcription in vitro by bis(quinone diimine) complexes of rhodium(III) and ruthenium(II). *Inorganic Chemistry*. **42** (3), 878-84 (2003).

19. Sorasaenee, K., Fu, P.K.-L., Angeles-Boza, A.M., Dunbar, K.R., Turro, C. Inhibition of Transcription in Vitro by Anticancer Active Dirhodium(II) Complexes. *Inorg. Chem.* **42** (4), 1267-1271, doi: 10.1021/ic020591p (2003).

20. Aguirre, J.D., Lutterman, D.A., Angeles-Boza, A.M., Dunbar, K.R., Turro, C. Effect of axial coordination on the electronic structure and biological activity of dirhodium(II,II) complexes. *Inorganic Chemistry*. **46** (18), 7494-502 (2007).

21. Raja, N.S., Nair, B.U. Chromium(III) complexes inhibit transcription factors

binding to DNA and associated gene expression. *Toxicology*. **251** (1-3), 61-5, doi: 10.1016/j.tox.2008.07.052 (2008).

22. Gao, F., Chen, X., Wang, J.Q., Chen, Y., Chao, H., Ji, L.N. In Vitro Transcription Inhibition by Ruthenium(II) Polypyridyl Complexes with Electropositive Ancillary Ligands. *Inorganic Chemistry*. **48** (13), 5599-601, doi: 10.1021/ic900902f (2009).

23. Chen, X., Gao, F., Zhou, Z.X., Yang, W.Y., Guo, L.T., Ji, L.N. Effect of ancillary ligands on the topoisomerases II and transcription inhibition activity of polypyridyl ruthenium(II) complexes. *Journal of Inorganic Biochemistry*. **104** (5) 576-82, doi: 10.1016/j.jinorgbio.2010.01.010 (2010).

24. Chen, X., Gao, F., Yang, W.Y., Sun, J., Zhou, Z.X., Ji, L.N. Effects of intercalative ligands on the DNA binding, DNA topoisomerase II and DNA transcription inhibition of polypyridyl ruthenium(II) complexes. **378** (1), 140-147, doi: 10.1016/j.ica.2011.08.047 (2011).

25. Chen, X., Gao, F., Yang, W.Y., Zhou, Z.X., Lin, J.Q., Ji, L.N. Structure-activity relationship of polypyridyl ruthenium(II) complexes as DNA intercalators, DNA photocleavage reagents, and DNA topoisomerase and RNA polymerase inhibitors. *Chemistry & Biodiveristy*. **10** (3), 367-84, doi: 10.1002/cbdv.201100414 (2013).

26. Chifotides, H.T., Fu, P.K., Dunbar, K.R., Turro, C. Effect of equatorial ligands of dirhodium(II,II) complexes on the efficiency and mechanism of transcription inhibition in vitro. *Inorganic Chemistry*. **43** (3), 1175-83 (2004).

27. Pauly, M., Kayser, I., Schmitz, M., Dicato, M., Del Guerzo, A., Kolber, I., Moucheron, C., Kirsch-De Mesmaeker, A. In vitro inhibition of gene transcription by novel photo-activated polyazaaromatic ruthenium(II) complexes. *Chemical Communications*. **10**, 1086-1087, doi: 10.1039/B202905G (2002).

28. Richardson, D.R. Iron and gallium increase iron uptake from transferring by human melanoma cells: Further examination of the ferric ammonium citrate-activated iron uptake process. *Biochimica et Biophysica Acta*. **1536** (1), 43-54, doi: 10.1016/S0925-4439(01)00034-5 (2001).

29. Collery, P., Keppler, B., Madoulet, C., Desoize, B. Gallium in cancer treatment. *Critical Reviews in Oncology / Hematology*. **42** (3), 283-296, doi: 10.1016/S1040-8428(01)00225-6 (2002).

30. Hedley, D.W., Tripp, E.H., Slowiaczek, P., Mann, G.J. Effect of gallium on DNA synthesis by human T-cell lymphoblasts. *Cancer Research*. **48** (11), 3014-8 (1988).

31. Chitambar, C.R., Narasimhan, J., Guy, J., Sem, D.S., O'Brien, W.J. Inhibition of

ribonucleotide reductase by gallium in murine leukemic L1210 cells. *Cancer Research*. **51**, 6199-6201 (1991).

32. Seidman, A.D., *et al*. Continuous infusion gallium nitrate for patients with advanced refractory urothelial tract tumors. *Cancer*. **68** (12), 2561-5 (1991).

33. Chitambar, C.R. Medical applications and toxicities of gallium compounds. *International Journal of Environmental Research and Public Health*. **7** (5), 2337-2361, doi: 10.3390/ijerph7052337 (2010).

34. Chitambar, C.R. Gallium-containing anticancer compounds. *Future Medicinal Chemistry*. **4** (10), 1257-1272, doi: 10.4155/fmc.12.69 (2012).

35. Trask, D.K., Muller, M.T. Stabilization of type I topoisomerase-DNA covalent complexes by actinomycin D. *Proceedings of the National Academy of Sciences of the United States of America*. **85** (5),1417-21, (1988).

36. Chang, T.C., Tsai, L.C., Hung, M.W., Chu, L.L., Chu, J.T., Chen, Y.C. Effects of transcription and translation inhibitors on a human gastric carcinoma cell line. Potential role of Bcl-X(S) in apoptosis triggered by these inhibitors. *Biochemical Pharmacology*. **53** (7), 969-77 (1997).

37. Mischo, H.E., Hemmerich, P., Grosse, F., Zhang, S. Actinomycin D induces histone gamma-H2AX foci and complex formation of gamma-H2AX with Ku70 and nuclear DNA helicase II. *The Journal of Biological Chemistry*. **280** (10), 9586-94 (2005).

38. Titov, D.V., *et al*. XPB, a subunit of TFIIH, is a target of the natural product triptolide. *Nature Chemical Biology*. **7** (3), 182-8, doi: 10.1038/nchembio.522 (2011).

39. Leuenroth, S.J., Crews, C.M. Triptolide-induced transcriptional arrest is associated with changes in nuclear substructure. *Cancer Researcg*. **68** (13) 5257-66, doi: 10.1158/0008-5472.CAN-07-6207 (2008).

40. Vispé, S., *et al*. Triptolide is an inhibitor of RNA polymerase I and II-dependent transcription leading predominantly to down-regulation of short-lived mRNA. *Molecular Cancer Therapeutics*. **8** (10), 2780-90, doi: 10.1158/1535-7163.MCT-09-0549 (2009).

41. MEGAscript T7 Transcription Kit User Guide [Internet]. Carlsbad: Life Technologies; 2012 [cited 2014 April 30]. Available from: http://tools.lifetechnologies.com/content/sfs/manuals/1330M_G.pdf

42. Voytas, D. Unit 2.5A Agarose Gel Electrophoresis. *Current Protocols in Molecular Biology*. 51:2.5A.1–2.5A.9, doi: 10.1002/0471142727.mb0205as51

(2001).

43. Fleige, S., Pfaffl, M.W., RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine*. **27** (2-3), 126-39, doi: 10.1016/j.mam.2005.12.003 (2006).

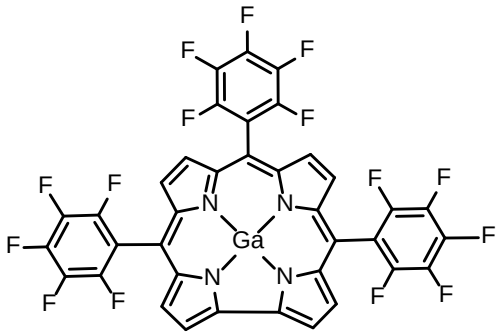
44. mini Quick Spin Columns Protocol [Internet]. Mannheim: Roche Diagnostics GmbH; 2013 [cited 2014 November 10]. Available from: https://pim-eservices.roche.com/LifeScience/Document/95c2b1f8-7cf1-e311-98a1-00215a9b0ba8

45. RNA quality control [Internet]. KU Leuven: Biomedical Genomics; 2007 [cited 2014 April 30]. Available from: http://biomedicalgenomics.org/RNA_quality_control.html

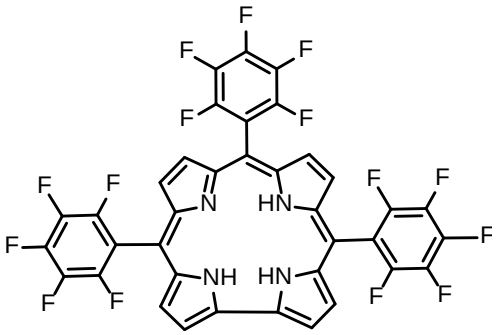
46. Sabnis, R.W. Handbook of biological dyes and stains: synthesis and industrial application. Hoboken, N.J.: Wiley. ISBN 978-0-470-40753-0. (2010).

Figure 1

Click here to download Figure: EditedFigure 1.pdf



Ga(tpfc)



tpfc

Figure 2

Click here to download Figure: Figure 2.pdf

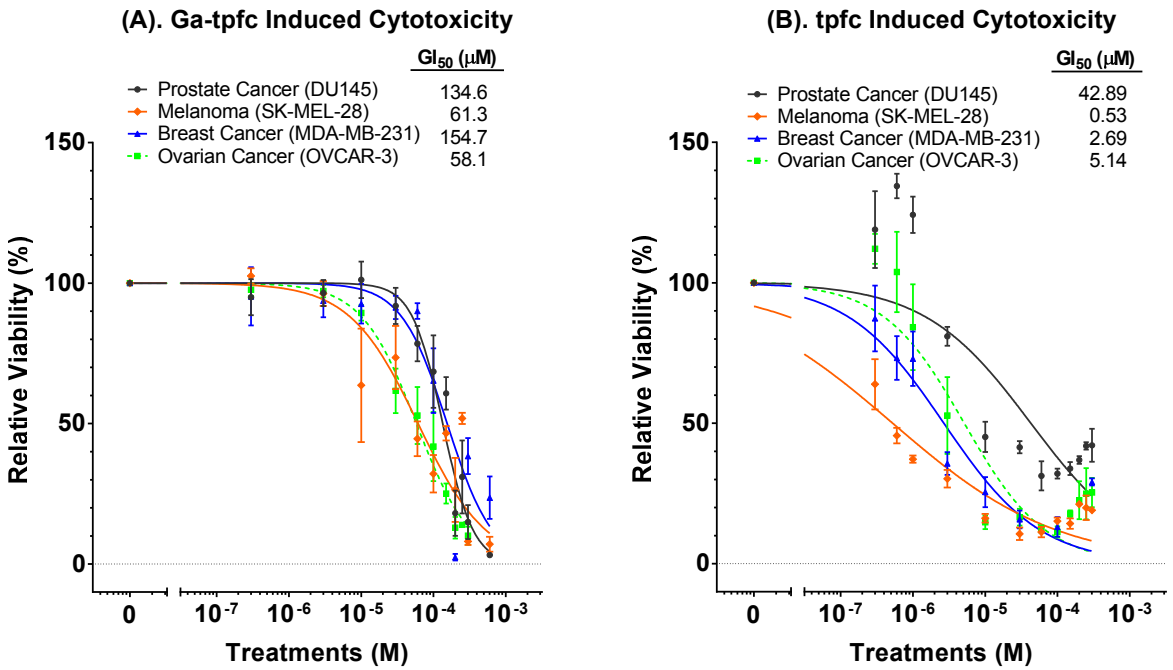


Figure 3
Click here to download Figure: Figure 3.docx

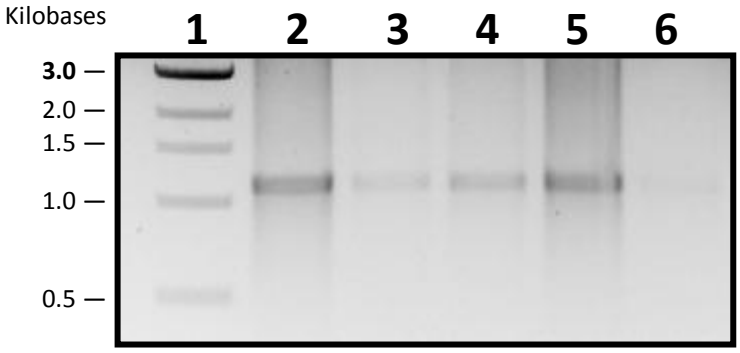


Figure 4
Click here to download Figure: Figure 4_final.docx

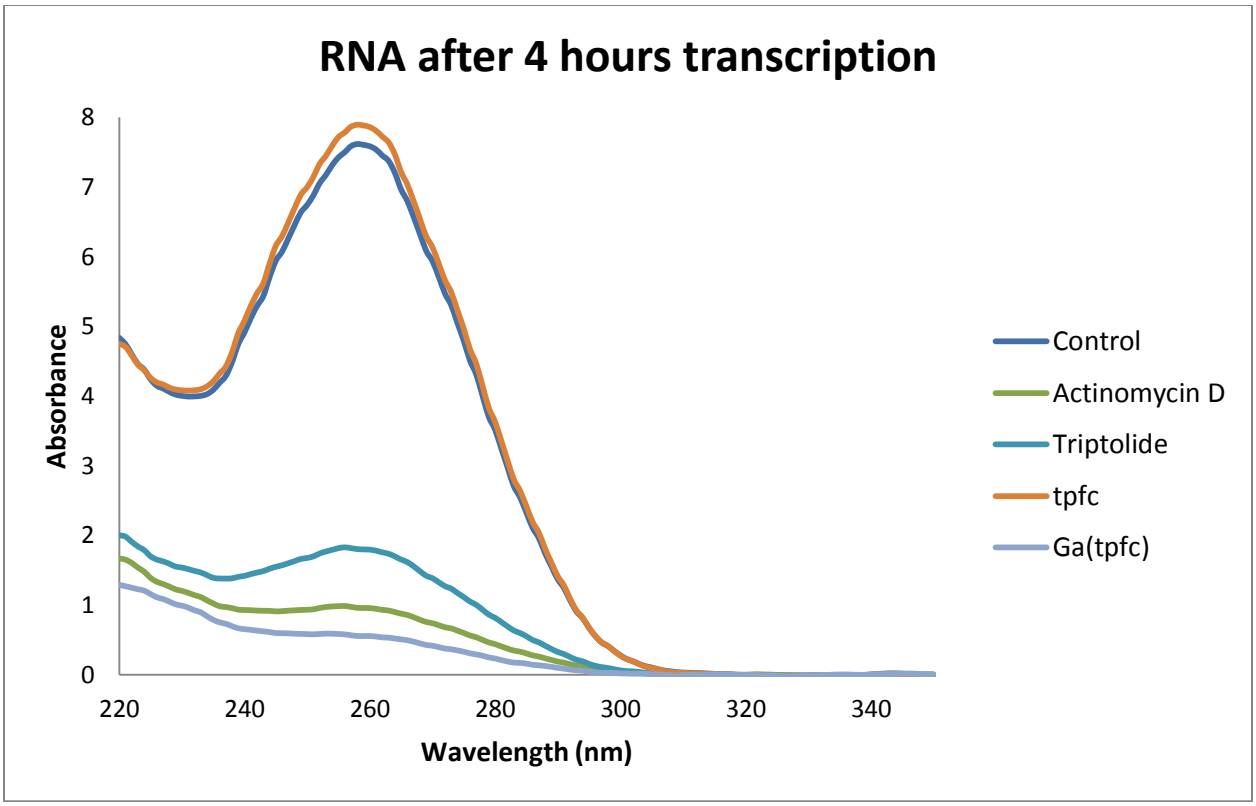


Table 1
Click here to download Table: Table 1 - final.xlsx

Thaw frozen reagents on ice:

75 mM solutions of adenosine triphosphate, guanosine triphosphate, cytidine triphosphate, and uridine triphosp
Nuclease-free Water
10x Reaction Buffer (1x Reaction Buffer: 40 mM Tris-HCl, 6 mM MgCl2, 2 mM spermidine, 10 mM dithiothreitol, p
Linearized template DNA (0.5 µg/mL, 1.85 kb)
RNA Polymerase Enzyme (20 U/µL)

hate (ATP, CTP, GTP, UTP)

ɔH 7.9)

Table 2
Click here to download Table: Table 2 - final.xlsx

To begin the transcription reactions, add the following amount of each reagent in 0.2 mL thin-walled PCR tubes:
2 µL ATP solution (75 mM)
2 µL CTP solution (75 mM)
2 µL GTP solution (75 mM)
2 µL UTP solution (75 mM)
1 µL nuclease-free water
2 µL 10X Reaction Buffer
2 µL of 0.5 µg/µL linear template DNA
5µL complex (nucleus-free water as the blank, actinomycin D, triptolide, tpfc, Ga(tpfc))
2 µL RNA Polymerase (20 U/µL)

Table 3
Click here to download Table: Table 3.xlsx

Time Point (hr)	Complex	A_{260}	A_{280}	A_{260}/A_{280}	Dilution Factor
4	Control	7.583	3.516	2.16	200
4	Actinomycin D	0.955	0.438	2.18	200
4	triptolide	1.794	0.816	2.2	200
4	tpfc	7.858	3.631	2.16	200
4	Ga(tpfc)	0.554	0.232	2.39	200

Table 3.

[RNA]:A260 (µg/ml)	[RNA] (mg/mL)
40	60.67
40	7.638
40	14.35
40	62.87
40	4.434

Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Table of Specific Materials and Equipment.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Actinomycin D	Sigma-Aldrich	A1410	Store at 2-8 °C , protect from light
Triptolide	Sigma-Aldrich	T3652	Store at 2-8 °C , protect from light
nuclease-free H_2O	Life Technologies	AM9938	
MEGAscript T7 Transcription Kit	Life Technologies	AM1334	Store at –20 °C
Ethidium Bromide	Sigma-Aldrich	E7637	CAUTION: For proper handling procedur
Tris Acetate	Sigma-Aldrich	T6025	
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	EDS	
UltraPure Agarose	Life Technologies	16500-100	
mini Quick Spin RNA Columns	Roche Life Science	11814427001	Store at 2-8 °C , do not freeze
1 kb DNA Ladder	New England Biolabs	N3232S	Store at –20 °C

es of ethidium bromide, please see: http://www.sciencelab.com/msds.php?msdsId=9927667

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: An in vitro Enzymatic Assay to Measure Transcription Inhibition by Gallium (III) and H3 5,10,15-tris(pentafluorophenyl)corroles

Author(s): Melanie A. Pribisko

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[ ] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Melanie Pribisko Yen

Department: Department of Chemistry

Institution: California Institute of Technology

Article Title: An in vitro Enzymatic Assay to Measure Transcription Inhibition by Gallium (III) and H3 5,10,15-tris(pentafluorophenyl)corroles

Signature: [signature] Date: 5/20/14

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

CALIFORNIA INSTITUTE OF TECHNOLOGY

Division of Chemistry and Chemical Engineering

Dr. Melanie Pribisko. Ph.D.
1200 E. California Blvd.
MC 127-72
E-mail: melaniep@caltech.edu

October 13, 2014

JoVE Submission No.: 52355R2
Title: An *in vitro* Enzymatic Assay to Measure Transcription Inhibition by Gallium(III) and H_3 5,10,15-tris(pentafluorophenyl)corroles

Dr. Sephora Zaman, Ph.D.
Science Editor, JoVE
1 Alewife Center
Suite 200
Cambridge, MA 02140

Re: JoVE Submission No.: 52355R2

Dear Dr. Zaman,

We thank the reviewers for thoroughly reading our manuscript and for their constructive comments. We have made several substantive changes to the manuscript in response to the reviewers' comments. In particular, we have emphasized that our manuscript provides an initial comparison of two standard transcription inhibitors compared to two cytotoxic corrole complexes, yet this experiment does not provide DNA binding information. We also clarified some of the language and incorporated more references, as requested by the reviewers. The details of the changes are described below:

Reviewer Comments #1 & #2:
The Introduction is very superficial with many incorrect and confusing statements and does not justify using Ga complex in the reported assay. For example line 117 contradicts line 118. Then line 104 contradicts line 106. (Reviewer #1)

Insufficient references are provided for listed statements. For Introduction, reference 11 alone seems insufficient for cover the progress in the field of transcription inhibition by metal coordination compounds. For a more objective introduction, the later important work by Dunbar [Inorg. Chem. 2007, 46, 7494-7502] for Rh complexes, and some Cr complexes by Nair [Toxicology 2008, 251, 61-65] and Ru complexes by Ji [Inorg. Chem. 2009, 48, 5599-5601; J. Inorg. Biochem. 2010, 104, 576-582; Inorg. Chim. Acta 2011, 378, 140-147; Chem. Biodivers 2013, 10, 367-384] should be concluded. If the authors just want to mention the first work in this field, [Inorg. Chem. 2003, 42, 878-884] should be used instead. Over the decade, the mechanism and structure-activity relationship have been thoroughly studied. These studies have become rather routine works. (Reviewer #2)

Response: Thank you very much for your suggestions. We have read through all of your suggested articles and have incorporated many into the manuscript. Corroles were used in this experiment to give a variety of transcription inhibitors, and also because Gallium complexes have been considered for anticancer therapeutics and have undergone clinical trials against hepatoma, lymphoma, bladder cancers, and other diseases, making them interesting to cancer researchers. After platinum, gallium is the second most promising metal to be considered for anticancer therapeutics and has also undergone many studies and investigations. Nitrate and chloride gallium salts have been evaluated in clinical trials against hepatoma, lymphoma, bladder cancers, and other diseases. Thank you very much for your comments; it is important that we justify the use of the Ga complex. This has been clarified in the manuscript and supported by the following references:

1. Chitambar, C.R., Narasimhan, J., Guy, J., Sem, D.S., O'Brien, W.J. Inhibition of ribonucleotide reductase by gallium in murine leukemic L1210 cells. *Cancer Research*. **51**, 6199-6201 (1991).
2. Collery, P., Keppler, B., Madoulet, C., Desoize, B. Gallium in cancer treatment. *Critical Reviews in Oncology / Hematology*. **42** (3), 283-296, doi: 10.1016/S1040-8428(01)00225-6 (2002).
3. Chitambar, C.R. Medical applications and toxicities of gallium compounds. *International Journal of Environmental Research and Public Health*. **7** (5), 2337-2361, doi: 10.3390/ijerph7052337 (2010).
4. Chitambar, C.R. Gallium-containing anticancer compounds. *Future Medicinal Chemistry*. **4** (10), 1257-1272, doi: 10.4155/fmc.12.69 (2012).
5. Hedley, D.W., Tripp, E.H., Slowiaczek, P., Mann, G.J. Effect of gallium on DNA synthesis by human T-cell lymphoblasts. *Cancer Research*. **48** (11), 3014-8 (1988).
6. Seidman, A.D., Scher, H.I., Heinemann, M.H., Bajorin, D.F., Sternberg, C.N., Dershaw, D.D., Silverberg, M., Bosl, G.J. Continuous infusion gallium nitrate for patients with advanced refractory urothelial tract tumors. *Cancer*. **68** (12), 2561-5 (1991).

The manuscript as a whole has undergone changes in the language to improve clarity. For instance, regarding lines 104 and 106: functionalization of the scaffold does cause unique shifts in absorbance, which allows us to characterize the molecule, but its photophysical properties are not inherently affected. Lines 117 and 118 are referring to different cancer cell lines. We have adjusted the grammar to clarify this comparison.

Reviewer Comment #2: The knowledge on inhibition of DNA transcription in direct interaction with DNA may not have anything to do with effect of metal complexes on transcription. One may get the idea, based on this manuscript, that the methodology used readily translates to way of the action of metal complexes. Metals are redox active and thus many metal complexes work on level of redox-based metabolic pathways. Authors should indicate if the Ga complex studied gets into nucleus, any PK data available.

Response: Ga(III) is redox inactive, and therefore is not directly involved in redox-based metabolic pathways. Regardless, Ga(III) does exhibit cytotoxicity and has been investigated for therapeutic purposes. The purpose of this paper is to show that the RNA transcription inhibition assay is an affordable and versatile method to assess whether potential anticancer molecules merit further testing. The particular experiment in the paper was not meant to imply the mechanism of the inhibition; we suggested in the discussion section that there are many different ways which transcription inhibition could occur, and that additional experiments would be needed for full mechanistic understanding. This has been clarified in the manuscript, with emphasis on the following references:

1. Lim, P., Mahammed, A., Okun, Z., Saltsman, I., Gross, Z., Gray, H.B., Termini, J. Differential cytostatic and cytotoxic action of metallocorroles against human cancer cells: Potential platforms for anticancer drug development. *Chemical Research in Toxicology*.

25 (2), 400-409, doi: 10.1021/tx200452w (2012).
2. Richardson, D.R. Iron and gallium increase iron uptake from transferring by human melanoma cells: Further examination of the ferric ammonium citrate-activated iron uptake process. *Biochimica et Biophysica Acta*. **1536** (1), 43-54, doi: 10.1016/S0925-4439(01)00034-5 (2001).

In the paper by Lim, et al., the studies show that each derivative of the metal corrole complex exhibits differential cytotoxicity towards human cells; for example sulfonated Ga corroles penetrate into the nucleus of the DU-145 cancer cell line, but do not do so for any of the other cancer cell lines tested in that study. In our paper, we focus on the general use of a transcription inhibition assay and not on nuclear penetration.

Reviewer Comment #3: The authors should pay more attention in the material and method section. For example, the recipe of TAE 50 X (section 3.2) isn't correct.

Response: The recipe for TAE 50x has been corrected to 2.0 M Tris-acetate and 0.05M EDTA. Thank you for pointing that out. We have reexamined each step in the materials and methods section and confirmed the accuracy.

Reviewer Comment #4: Line 181: Procedure of changing cis-[Pt(NH3)2Cl2] to cis-[Pt(NH3)2(H2O)2]2+ is not correct. As found in my own experience, this procedure is too easy to get completely converted hydrate. Then I changed to use the following procedure: "Activated cisplatin, cis-[Pt(NH3)2(OH2)2]2+, was prepared by reacting an aqueous solution of cisplatin with 2 equiv of AgNO3 for 1 day at room temperature in the dark. The reaction mixture was centrifuged for 10 min to remove AgCl. The aqueous layer was separated from the precipitate, and the solution was centrifuged for another 10 min; this process was repeated twice. [Inorg. Chem. 2004, 43, 1175-1183]" It should be noted that cisplatin must be handled avoiding light (this kind of notice can be found on the bottle of any cisplatin, solid or solution). If we stir it in water in light for several hours, as suggested here, only waste residue of cisplatin will be left, rather than activated one.

Response: First, we would like to clarify that in the original procedure, all platinum work was carried out in the dark. We regret not making that clear at the time. Second, we would like to thank the reviewer for providing experimental procedures from his/her own work. Third, we tested the procedure recommended by the reviewer, which differed from our original procedure only by the separation method (filtration vs. centrifugation).

When we redid the experiment using the explicit instructions for the preparation of cisplatin provided by Reviewer #2, we observed inconsistent results. As I mentioned in previous correspondence with the JoVE editor, we included two known transcription inhibitors in this study since the reviewers had concerns with the cisplatin. We decided to remove cisplatin as a known transcription inhibitor and only present data for the Actinomycin D and triptolide inhibitors. Ultimately, we came to this conclusion for three reasons: 1. of all the samples, only the cisplatin required advance preparation and purification. Setting the reaction up 24 hours in advance and using it within a small timeframe may not be convenient for users of the protocol. 2. the cisplatin reaction and purification needs to be carried out in the dark. Since I already work with light-sensitive compounds, my lab is set up for this work. In fact, my hood sash has a filter that blocks 99% of UV light. We cannot assume all labs and/or coworkers can tolerate working in the dark. 3. since the reaction is time and light sensitive, it is difficult to quantify the yield. We assume 100% conversion to active platinum, but that may not be the case and characterization is not easy. Since this is a protocol paper, we want it to be a resource with extremely reliable results and have taken the steps to do so.

Reviewer Comment #5: Line 191: "Note: ... Small amounts of DMSO (up to 1%) do not induce cytotoxicity in the cells (unpublished data)." Please be noted that this depiction is very dangerous. Dimethyl sulfoxide (1-10%) has been shown to accelerate strand renaturation and is

believed to give the nucleic acid thermal stability against denaturation. That's why DMSO is used as a PCR cosolvent, helping improve yields, especially in long PCR. Therefore, "do not induce cytotoxicity" doesn't mean do not affect DNA transcription. If DMSO affects transcription but not tumor cell activity, the conclusion might be "inhibition of DNA transcription is not relative to antitumor activity".

Response: In the manuscript, “up to 1%” meant “less than 1%”. We acknowledge the importance of using less than 1% DMSO and have edited the text to more precise language.

Reviewer Comment #6: Line 225: Lithium chloride precipitation. This part is somewhat not necessary because DNA and DNA polymerase do not really affect the result of RNA quantification by gel electrophoresis. If one really wants to quantify RNA by UV, a RNA spin column could get over 90 % RNA from mixture of DNA and protein within 2 minutes, while precipitation method may give apparently lower recovery and purity and is time cost.

Response: We believe it is important to purify the transcription reaction for two reasons: 1. it stops the transcription reaction, preventing error being introduced due to different reaction time lengths, and preventing any degradation of the RNA product from the enzymes in the solution. and 2. we found gels run after a purification step to be much clearer than gels run with all the reaction components. We redid the experiment using mini Quick Spin RNA Columns to purify the RNA, and we have included that change in our updated procedure.

Reviewer Comments #7 and #8: Figure legends have insufficient information, for example in Figures 2 and 3 no information about concentration of Ga complex is given (Reviewer #1).

Also, it should be considered to improve the figure legends, especially the one of figure 3, that doesn't explain well the differences between sample 1-4 and 5-8 (Reviewer #3).

Response: All figure legends and captions have been edited for maximum clarity. In Figure 2, the concentration is reflected in the x-axis labeled “Treatments”, and is measured on a logarithmic scale in moles. In Figure 3, the ratio of [metal complex] to [DNA] was 0.01. Since there was 0.43 pmol DNA, all inhibitors are at 4.3 fmol concentration.

Reviewer Comment #7: Figure 3. Please show the whole picture of gel with ladder (size marker).

Response: Figure 3 has been changed to include the DNA ladder, and modified to improve clarity.

Reviewer Comment #8: Molar absorptivities are needed for uv/vis spectra.

Response: We have corrected this oversight.

We hope that these changes to the manuscript satisfy both the reviewers and the editorial board at JoVE. We wish to publish an article of high quality that will be of interest to researchers in the biological and chemical sciences, therefore, please let us know if there is anything else we can do to improve the content of our article.

Sincerely,

Melanie Pribisko

Supplemental File (as requested by JoVE) - Rotary Evaporation

Click here to download Supplemental File (as requested by JoVE): Rotary Evaporation_MAP.docx