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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