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Functionalized Spirocyclic Heterocycle Synthesis and Cytotoxicity Assay

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TITLE:

Functionalized Spirocyclic Heterocycle Synthesis and Cytotoxicity Assay

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KEYWORDS:

MTT assay, spirocyclic oximes, heterocycles, cytotoxicity, solid-phase synthesis, cell viability, colorimetric assay

SUMMARY:

Here, we describe a bioassay using 3-(4',5'-dimethylthiazol-2'-yl)-2,5- diphenyltetrazolium bromide (MTT) to test previously synthesized spirocyclic oximes.

ABSTRACT:

Spirocyclic heterocycles have recently been reported in literature to be potential drugs for cancer therapy. The synthesis of these novel orthogonal ring systems is challenging. An efficient methodology to synthesize these compounds was recently published that described the solid phase synthesis in four steps rather than the previously reported five steps. The advantage of this shorter synthesis is the elimination of the use of toxic reagents. Low-loading Regenerating Michael (REM) linker-based resin was found to be crucial in the synthesis as high-loading versions prevented the addition of reagents containing bulky phenyl and aromatic side chains. The colorimetric 3-(4',5'-dimethylthiazol-2'-yl)-2,5- diphenyltetrazolium bromide (MTT) assay was used to examine the cytotoxicity of micromolar concentrations of these novel spirocyclic

molecules in vitro. MTT is readily available commercially and produces relatively fast, reliable results, making this assay ideal for these spirocyclic heterocycles. Orthogonal ring structures as well as furfurylamine (a precursor in the synthesis method containing a similar 5-member ring motif) were tested.

INTRODUCTION:

Small-molecule inhibition of the interaction of E3 ubiquitin-ligase mouse double minute 2 homolog (MDM2) with p53 is known to restore p53-mediated induction of tumor cell apoptosis[1-3]. MDM2 is a negative regulator of the p53 pathway and is often overexpressed in cancer cells[4-9]. Recent crystallographic and biochemical studies have revealed that small molecules containing a spirocyclic framework can effectively inhibit MDM2-p53 interactions[10]. The spirocyclic framework (**Figure 1**, shaded in blue) is considered a privileged motif as derivatization of this rigid orthogonal ring system has led to the discovery of novel therapeutic drugs. Accessing this interesting architecture poses a challenge when using traditional organic synthesis techniques. Although the therapeutic effects of spirocyclic molecules in biological systems have been investigated, synthesis of these molecules is still a cumbersome process. Unwanted side products, using harsh conditions, and hazardous transition metals are often problematic.

The potential use of the spirocyclic motif in drug development led to the development of a protocol utilizing solid-phase synthesis to generate a library of molecules with the motif in addition to other interchangeable functional groups[11,12]. The separation of products and reactants between steps could be achieved by simply utilizing an REM linker attached to a resin bead and a solid-phase filter vessel. This would cut down steps and potentially increase yields. This synthetic approach could produce a large array of potential drug candidates. However, the effectiveness of these molecules in a biological system would require further investigation.

To determine the cytotoxicity of these spirocyclic compounds, the MTT assay[13,14] was employed. This method measures cell viability and can be used to indirectly determine cell cytotoxicity. Different concentrations of the inhibitors were added to cultured cells in a 96-well plate, and the proportion of living cells was measured by colorimetric analysis of the extent of reduction of yellow MTT by mitochondrial dehydrogenases to the purple formazan compound (**Figure 2**). The activity is most often reported as an IC_{50} value—the concentration at which cell growth is inhibited by 50% relative to an untreated control. This paper describes the protocol for the MTT assay and the preliminary results of these novel spirocyclic molecules.

PROTOCOL:

NOTE: Several chemicals and biological reagents used in this protocol are toxic and carcinogenic. Consult relevant material safety data sheets (MSDS) prior to use. Use appropriate personal protective gears (Occupational Safety and Health Administration-approved safety googles, proper gloves, lab coats, full-length pants, and closed-toe shoes) prior to starting the experiment.

In addition, adopt appropriate safety practices when performing synthesis and handling toxic chemicals and reagents (fume hood).

1. Solid phase synthesis of spirocyclic heterocycles 6 and 7

NOTE: Synthesis was based on previously published work[11,12]. The updated protocol reveals that the tetrabutylammonium fluoride-catalyzed ring opening of the tricyclic heterocycle was not needed, and thus its elimination shortens the synthetic procedure.

1.1. Perform Michael addition of furfurylamine to the REM linker (duration: 25 min setup + 24 h reaction time).

1.1.1. Add 1 g (1 equivalents [equiv.]) of REM resin, 20 mL (20 equiv.) of dimethylformamide (DMF), and 2.4 mL of furfurylamine to a 25 mL solid-phase reaction vessel. Agitate the reaction vessel at room temperature for 24 h following the reaction initiation.

NOTE: Ensure thorough mixing so that the resin does not sit at the bottom of the vessel.

1.1.2. Wash the resin with DMF 1x after the reaction is complete. Then, wash 4x, alternating between dichloromethane (DCM) and methanol. Dry the resin thoroughly in the reaction vessel following washes.

1.2. Perform tandem Michael addition/1,3-dipolar cycloaddition (duration: 25 min setup + 48 h reaction time).

1.2.1. To the dry resin, add 1.48 mL (5 equiv.) of triethylamine (TEA), 0.637 g (2 equiv.) of nitro-olefin, and 10 mL of dry toluene to the reaction vessel.

1.2.2. Then, add 1.085 mL (4 equiv.) of trimethylsilyl chloride (TMSCl) to the reaction vessel in a well-ventilated fume hood.

NOTE: As this reaction produces HCl gas, do not cap the reaction vessel until the gas has been released under a fume hood.

1.2.3. Securely cap the reaction vessel, and agitate at room temperature for 48 h.

NOTE: Ensure thorough mixing of the resin with the reagents.

1.2.4. Use 5 mL of methanol to quench the reaction.

1.2.5. Drain the vessel to remove the solution, and then wash 4x, alternating between DCM and methanol. Dry the resin thoroughly in the reaction vessel following washes.

1.3. Perform *N*-alkylation of the resin-bound heterocycle to form the quaternary amine (duration: 10 min setup + 24 h reaction time).

1.3.1. To the dry resin in the reaction vessel, add 5 mL of DMF and 10 equiv. of alkyl halide, and agitate at room temperature for 24 h.

NOTE: Ensure thorough mixing of the reagents with the resin.

1.3.2. Wash the resin with DMF 1x after the reaction is complete. Then, use DCM and methanol alternately to wash 4x. Dry the resin in the reaction vessel following washes.

1.4. Perform β-elimination of the quaternary amine for cleavage from the polymer support (duration: 15 min setup + 24 h reaction time).

1.4.1. To the dry resin in the reaction vessel, add 3 mL of DCM and 1.49 mL (5 equiv.) of TEA to cleave the heterocycle from the polymer support.

1.4.2. Agitate the reaction mixture for 24 h to ensure thorough mixing of the resin with the solution. Wash 4x, alternating between DCM and methanol. Collect the elution from all the washes, and concentrate via rotatory evaporation.

1.4.3. Triturate with methanol to purify the spirocyclic oxime. Dry the resin thoroughly in the reaction vessel following washes for reuse in future experiments.

2. Cytotoxicity assay using MTT[14]

2.1. Prepare 20 mL of a 5 mg/mL MTT solution using sterile phosphate-buffered saline (PBS, 0.9% NaCl in water) as the diluent. Filter and store at -20 °C. Then, prepare a 1:1 dilution of the MTT solution from step 2.1 in serum-free cell culture medium (DMEM).

2.2. Prepare 1 mL each of stock solutions in 1.5 mL microcentrifuge tubes of 100 mM, 10 mM, 1 mM, 100 µM, 10 µM, 1 µM, 0.1 µM, and 0.01 µM of test compounds in dimethyl sulfoxide (DMSO). Store at -20 °C. Prepare 200 µL per dose of the working solutions of test compounds by diluting stock concentrations 1:1000 in serum-free medium in 1.5 mL tubes.

2.3. In the tissue culture hood, seed COS-7 cells (African green monkey kidney cells, *Cercopithecus aethiops* kidney) in complete medium [DMEM with 10% fetal bovine serum (FBS)] onto flat-bottom, tissue-culture-treated 96-well plates at a concentration of 4×10^3 cells/200 µL per well using a multi-channel pipettor. COS-7 cells were chosen because (1) these are commonly used cells for cytotoxicity assays and (2) these were already available in the institution.

2.4. Incubate COS-7 cells for 24 h at 37 °C in an atmosphere containing 5% CO_2.

2.5. Aspirate the supernatant from the wells using a glass Pasteur pipette attached to a vacuum pump. Dose the cells in triplicate with the test compounds using the working solutions prepared in step 2.2 (See **Table 1**). Incubate cells as described in step 2.4.

2.6. Aspirate the supernatant from the wells. Add 200 µL of MTT solution to each well. Incubate at 37 °C in an atmosphere containing 5% CO_2 for 4 h.

2.7. Gently aspirate the supernatant from the wells without disturbing the purple formazan crystals. Add 200 µL of DMSO to each well to dissolve the purple formazan crystals. Incubate at room temperature for 15 min.

2.8. Measure absorbance at 590 nm[14] or 600 nm for each well using a 96-well plate reader. Use wells with no cells as background and average the absorbance value. Subtract the averaged absorbance background value from the absorbance value of each treated well. Normalize the data as a percentage of the average zero dosage value (average the three zero-dose values). Plot data on the y-axis: linear (% relative cell viability); x-axis: log (concentration). Plot each series as an individual curve (e.g., triplicate data should have 3 curves)

REPRESENTATIVE RESULTS:

Spirocyclic oximes **6** and **7** were synthesized using a modified protocol (**Figure 1**). Michael addition of furfurylamine to an REM linker **1b** afforded polymer-bound resin **2**. The progress of the reaction was monitored by infrared (IR) spectroscopy by detecting the disappearance of the α,β-unsaturated ester at 1722 cm^{-1} (**Figure 3**). Spirocyclic-bound resin **4** was formed from **2** via a transient intermediate **3**. Methanolic hydrolysis of **4** produced 3-[(*3E*)-(*2S, 4R*)-2-phenyl-3-hydroxyimino 4-hydroxymethyl-pyrrolidin-1-yl]-propionic acid methyl ester **7**, while alkylation followed by β-elimination afforded (*3E*)-(*2S, 4R*)-4-hydroxymethyl-1-methyl-2-phenyl-3-pyrrolidine oxime **6**. The identity of the spirocyclic oximes was determined by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopic analysis and the purity by mass spectroscopy based on our previous results[11].

The MTT assay is a well-known colorimetric assay for determining cell viability[12]. As seen in **Figure 2**, mitochondrial reductases present in living cells convert the yellow tetrazolium of MTT to an insoluble purple formazan solid. Using a spectrophotometer, the formazan formation is quantified by measuring the absorbance at 600 nm. Cisplatin, which is known to induce cell death at high concentrations, was used as a positive control (**Figure 4**). As expected, the higher the concentration of cisplatin, the lower is the cell viability. Next, the MTT assay was used to test the spirocyclic compounds **6** and **7** and furfurylamine. Furfurylamine was used to determine the effect of the furan ring alone compared to the spirocyclic framework. As depicted in **Figure 5**, furfurylamine and spirocyclic oxime **6** showed similar cytotoxicity. However, the toxicity of spirocyclic compound **7** was noticeably greater than that of furfurylamine and **6**. A library of spirocyclic oximes will be synthesized to fully investigate the cytotoxicity as well as the other anticancer effects of these heterocycles.

FIGURE AND TABLE LEGENDS:

Figure 1: Construction of spirocyclic compounds using an updated solid phase synthesis. The orthogonal spirocycic framework is shaded in blue. Note that step (c) is not needed, which avoids using the toxic reagent TBAF. The reaction conditions are as follows: (a) furfurylamine, DMF, (b) β-nitrostyrene, TMSCl, TEA, toluene, (c) TBAF, (d) alkyl halide, DMF, and (e) TEA, DCM. Abbreviations: TBAF = tetrabutylammonium fluoride; DMF = dimethylformamide; TMSCl = trimethylsilyl chloride; TEA = triethylamine; DCM = dichloromethane; ISOC = intramolecular silyoxy olefin cycloaddition.

Figure 2: Mechanism of the MTT assay. Visibly yellow tetrazolium salt of MTT is reduced by mitochondrial reductases in living COS-7 cells to form purple insoluble formazan. Abbreviation: MTT = 3-(4',5'-dimethylthiazol-2'-yl)-2,5- diphenyltetrazolium bromide.

Figure 3: Monitoring the progress of each solid phase reaction step by infrared spectroscopy. The stretching frequency at 1717 cm^{-1} indicated the presence of an unsaturated ester, 1733 cm^{-1} depicted a saturated ester, and signal around 3300–3500 cm^{-1} indicated the presence of a hydroxyl group. Detectable stretching frequencies for polystyrene are also shown. Abbreviations: REM = Regenerating Michael; ISOC = intramolecular silyoxy olefin cycloaddition.

Figure 4: Effects of cisplatin on COS-7 cell viability in a modified MTT assay. Concentrations of cisplatin ranged from 0 μM to 60 μM.

Figure 5: Effects of test compounds on COS-7 cell viability in a modified MTT assay. Concentrations ranged from 0 μM to 100 μM and were plotted on a log scale.

Table 1: Layout of the 96-well plate. All test data rows were in triplicate. Wells containing only COS-7 cells and medium were used as controls. To ensure that DMSO was not the cause of cytotoxicity in the cisplatin-dosed cells, wells containing only DMSO were used as solvent controls. Wells containing COS-7 cells are highlighted. Abbreviations: DMSO = dimethylsulfoxide; PBS = phosphate-buffered saline.

DISCUSSION:

The synthesis of the spirocyclic compounds was based on previous research conducted by this laboratory, but with some modifications (**Figure 1**)[11,12]. The progress of each reaction step was monitored by IR spectroscopy. Michael addition of the REM linker **1** with furfurylamine afforded polymer-bound **2** (IR 1722 cm^{-1} → 1731 cm^{-1}). From the previous report, ISOC of **2** produced the tricyclic heterocyclic compound **3**, as confirmed by the detection of the TMS group (IR 1214 cm^{-1}). This is a critical step of the synthesis as ISOC provided the necessary regio- and stereoselectivity of the products. A hydroxyl group stretching frequency of 3500 cm^{-1} was

observed instead of the frequency of the TMS functional group. This may be because the tricyclic compound is a transient intermediate that leads to the spirocyclic system.

Different types of REM resin were found to limit the synthesis. High-loading polymer (1.00 mmol/g) prevented the synthesis of spirocyclic compounds containing bulky R_2 side chains. Due to the similarities in the functional groups in resins **4** and **5**, the results of IR were inconclusive. The success of this step could only be determined by attempting to regenerate the REM linker (**5** → **1**). Regeneration did not occur in instances when a bulky R_2 group was added. Low-loading resins (0.5 mmol/g or lower) are recommended for successful synthesis. This synthesis method is consistent with procedures described in the literature.

As a preliminary test, a protocol was developed for a cytotoxicity assay using MTT. Over the course of several trials, critical steps and limitations were discovered. For the results to be normalized across all wells, cells had to be evenly seeded across wells, necessitating the measurement of the cell concentration prior to seeding. The assay required plates with flat-bottomed wells, as the absorbance could not be accurately read from round-bottomed wells. Additionally, excess MTT that remained after incubation had to be removed to prevent interference in the readings without disturbing the insoluble formazan.

The absorbance of the dissolved formazan should be read at 590 nm. However, current instrumentation in the lab necessitated taking readings at 600 nm instead. Storage at 0 °C was found to be important for the chemicals used in the assay (cisplatin, spirocyclic molecules, furfurylamine). DMSO—a chemical with known cytotoxicity—was used as the solvent for the test compounds and was used to make dilutions for the assay. The MTT reagent itself had to be prepared, as it was stored as a powder that needed to be dissolved and filtered, as insoluble particles interfered with readings.

Overall, the results for this assay are intended to be preliminary, as only a small number of molecules were tested. An exhaustive test with a battery of molecules is planned, and a full manuscript will be forthcoming. In addition, the synthesis might be applicable for amines derived from pyrrole-2-carbaldehyde. in which case, spirocyclic pyrrolidines can be synthesized and tested for cytotoxic effects on cancer cell lines.

ACKNOWLEDGEMENTS:

This work was funded by a grant from the Faculty Research Council to K.S.H. (Office of Research and Grants, Azusa Pacific University-USA). A.N.G. and J.F.M. are recipients of the Scholarly Undergraduate Research Experience (SURE) Fellowship. S.K.M. and B.M.R. are recipients of the STEM Research Fellowship Grants (Center for Research in Science, Azusa Pacific University-USA). We are grateful to Dr. Matthew Berezuk and Dr. Philip Cox for guidance on the bioassays.

DISCLOSURES:

The authors have nothing to disclose.

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14. Modified procedure from https://www.abcam.com/kits/mtt-assay-protocol

Figure 1: Construction of spirocyclic compounds using an updated solid phase synthesis.

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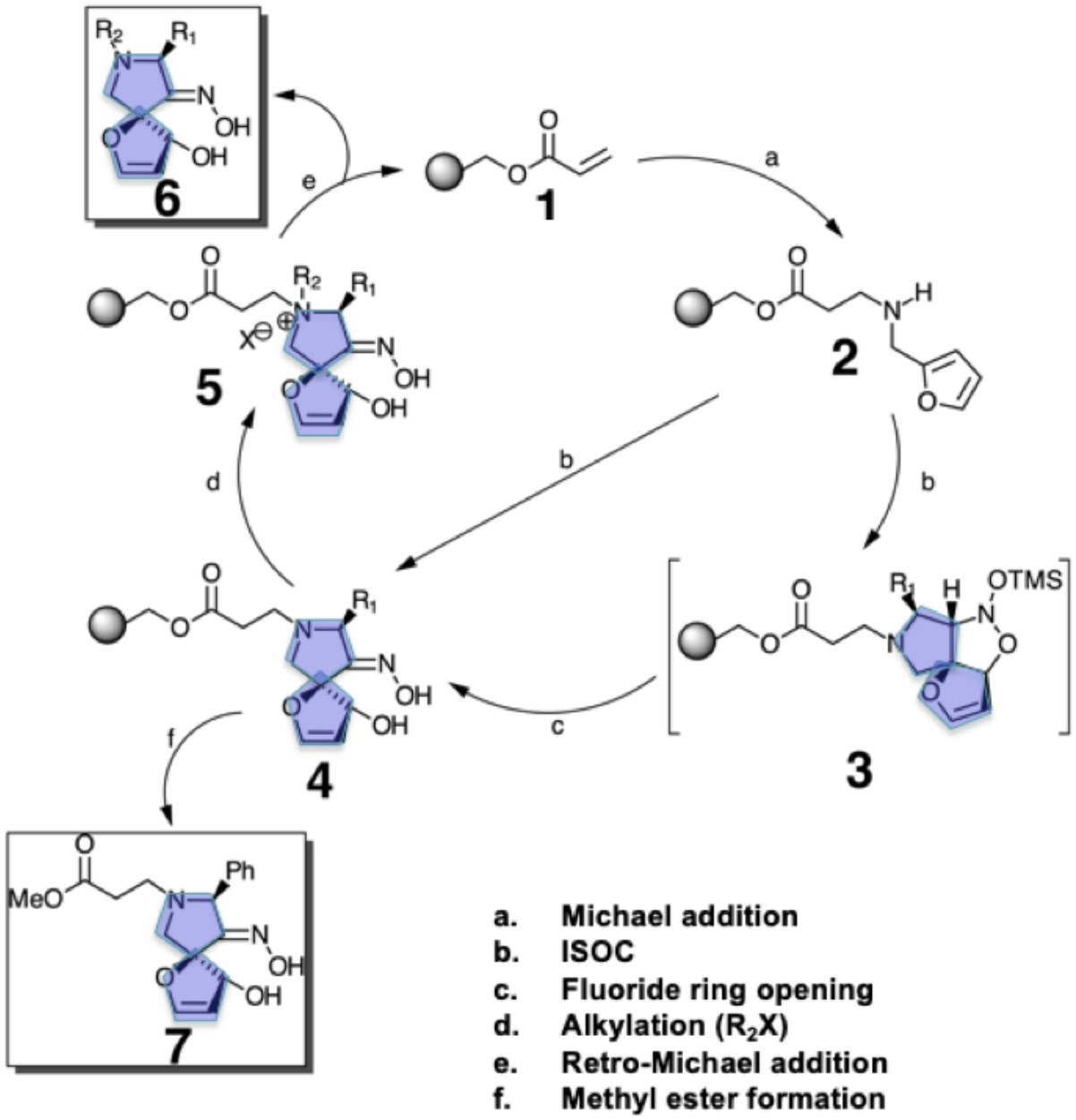

Figure 2: Non-cytotoxic dose determination by MTT assay.

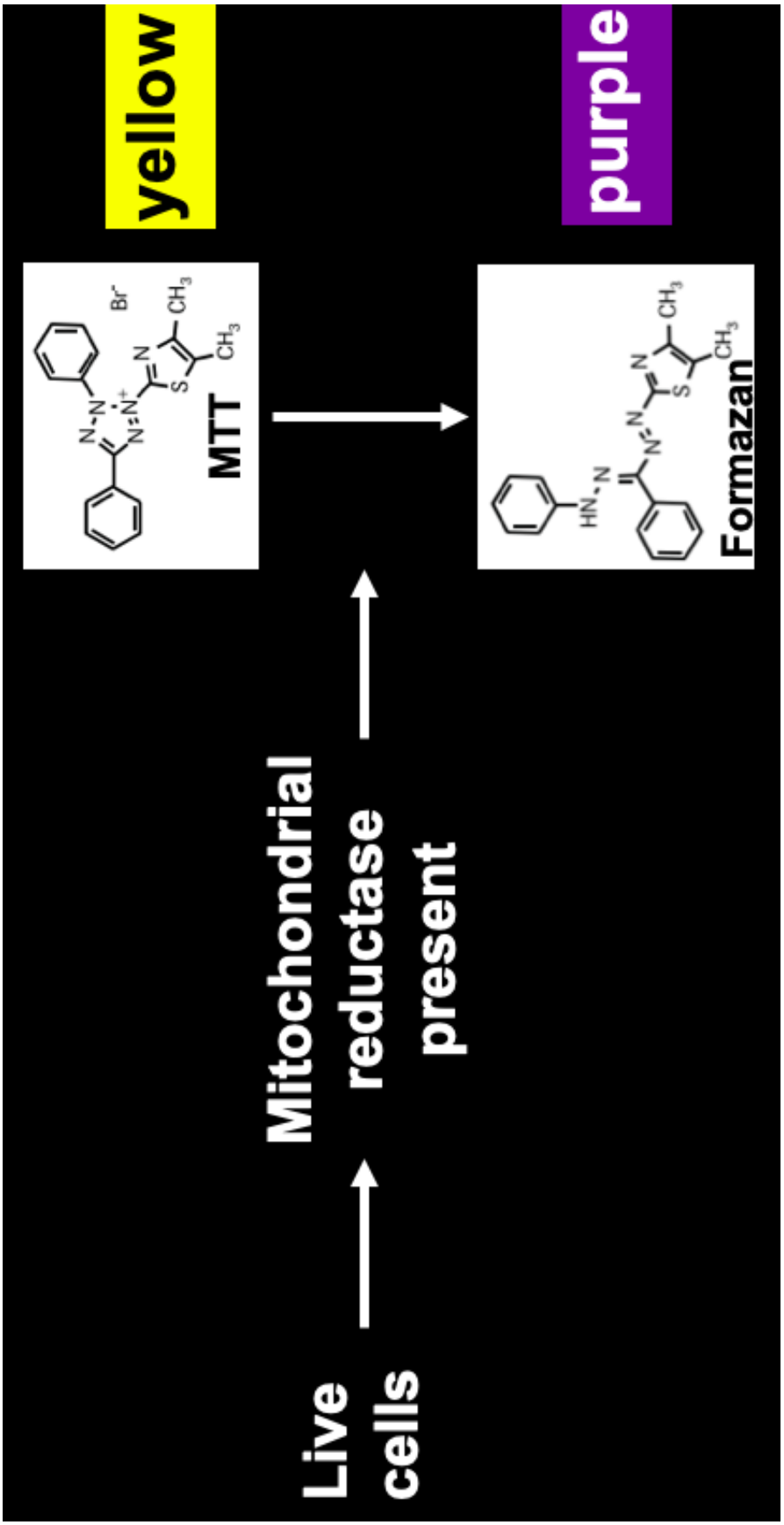

Figure 3: Monitoring the progress of each solid phase reaction step by Infra-red (IR) spectroscopy.

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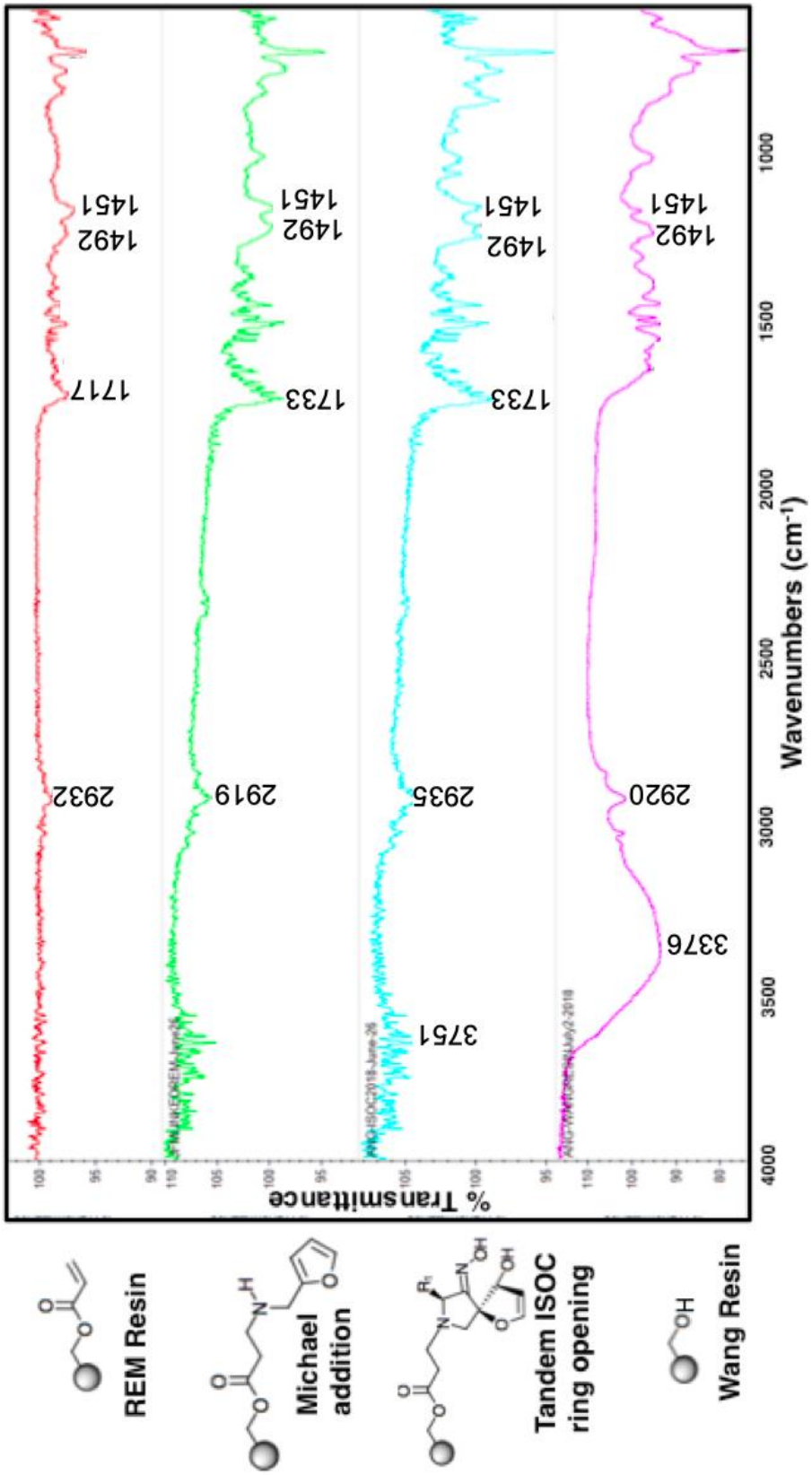

Figure 4: Cell viability in the presence of cisplatin using a modified MTT assay.

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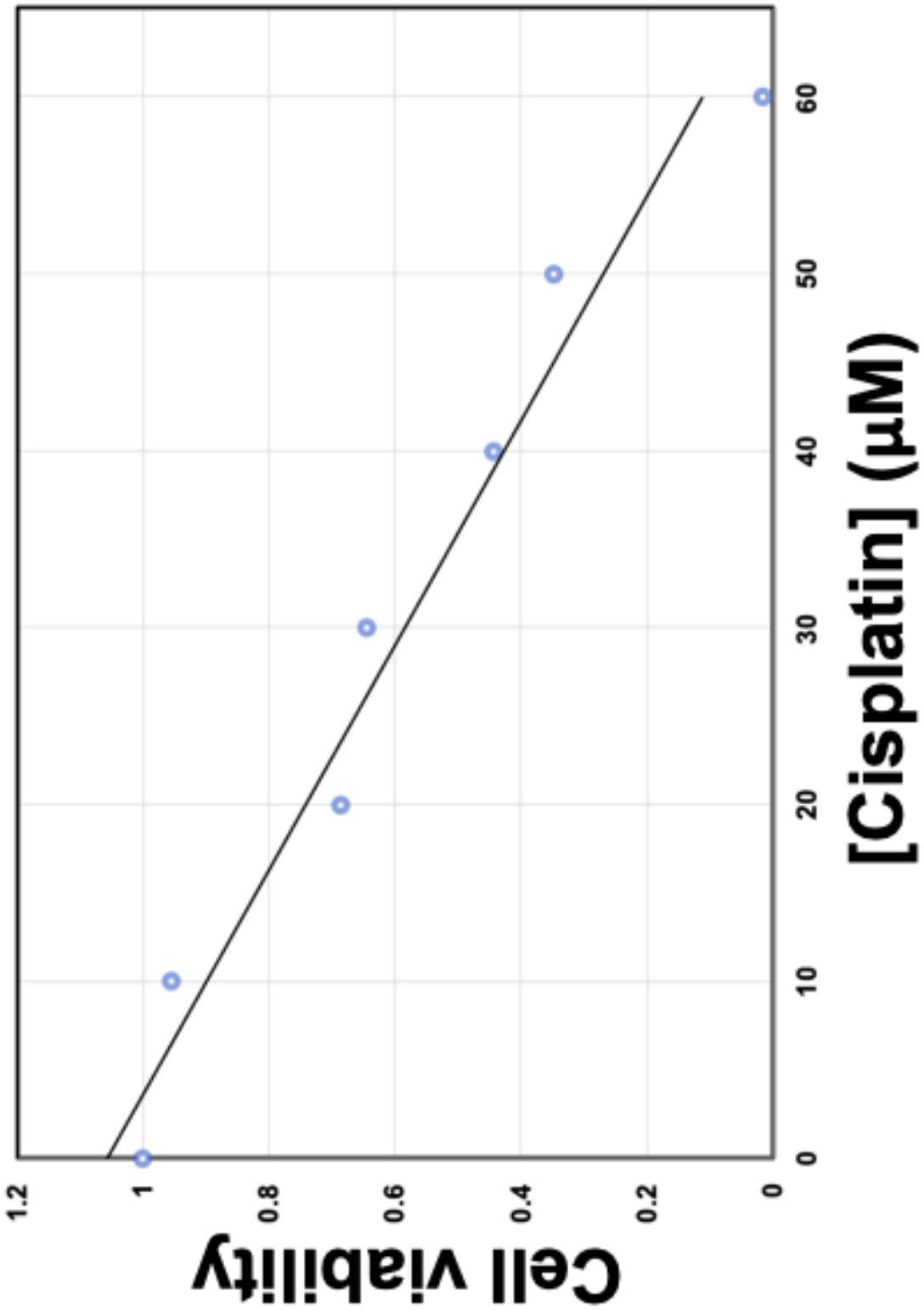

Figure 5: Cell viability in the presence of inhibitors using a modified MTT assay.

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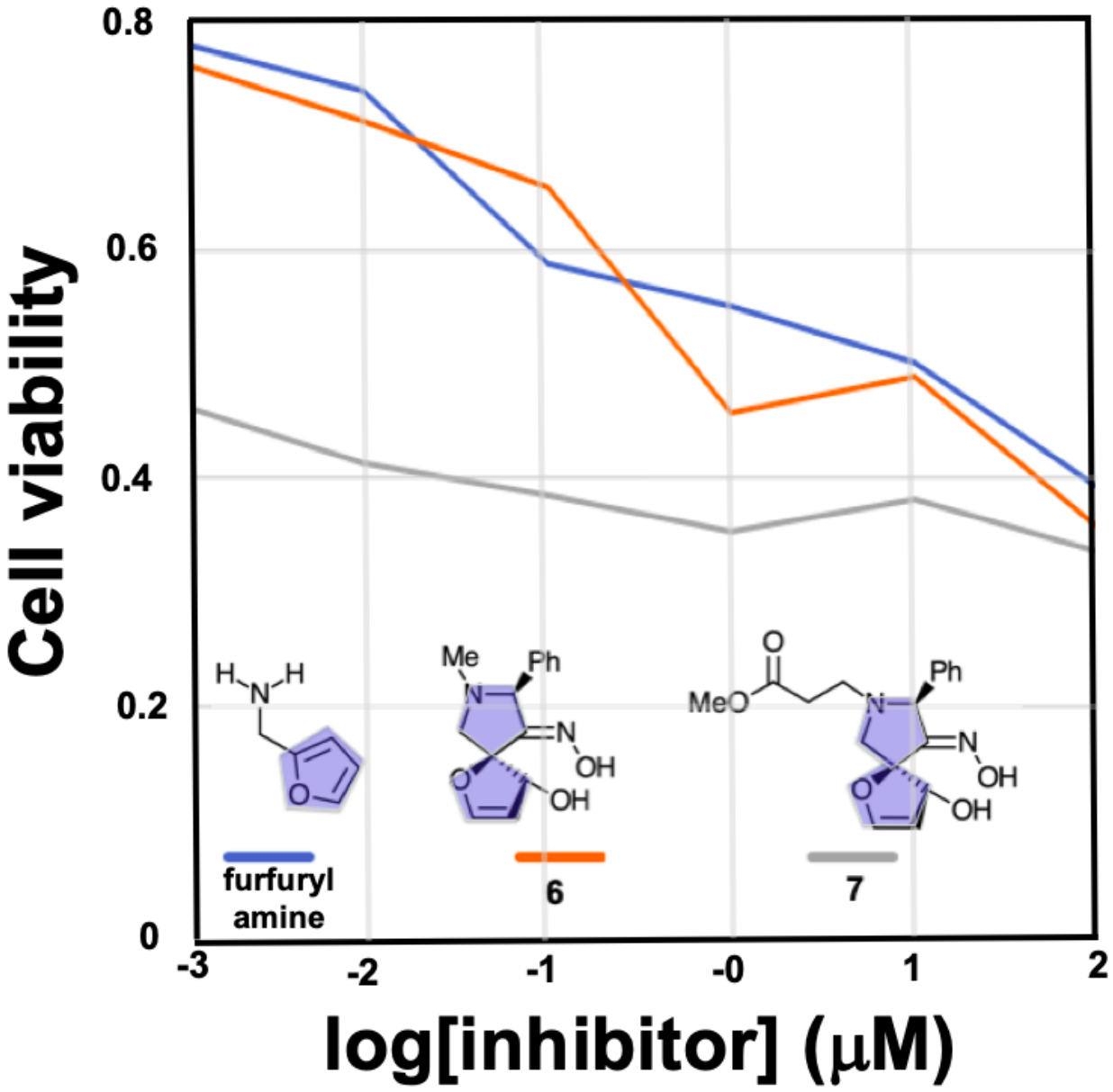

Table #1

PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
PBS	0 uM cisplatin	10 uM cisplatin	20 uM cisplatin	30 uM cisplatin	40 uM cisplatin	50 uM cisplatin	60 uM cisplatin	DMSO	Media	Media	PBS
PBS	0 uM cisplatin	10 uM cisplatin	20 uM cisplatin	30 uM cisplatin	40 uM cisplatin	50 uM cisplatin	60 uM cisplatin	DMSO	Media	Media	PBS
PBS	0 uM cisplatin	10 uM cisplatin	20 uM cisplatin	30 uM cisplatin	40 uM cisplatin	50 uM cisplatin	60 uM cisplatin	DMSO	Media	Media	PBS
PBS	0 uM inhibitor	0.001 uM inhibitor	0.01 uM inhibitor	0.1 uM inhibitor	1 uM inhibitor	10 uM inhibitor	100 uM inhibitor	Media	Media	Media	PBS
PBS	0 uM inhibitor	0.001 uM inhibitor	0.01 uM inhibitor	0.1 uM inhibitor	1 uM inhibitor	10 uM inhibitor	100 uM inhibitor	Media	Media	Media	PBS
PBS	0 uM inhibitor	0.001 uM inhibitor	0.01 uM inhibitor	0.1 uM inhibitor	1 uM inhibitor	10 uM inhibitor	100 uM inhibitor	Media	Media	Media	PBS
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
CELLS			
COS-7 cells (ATCC CRL-1651)	ATCC	CRL-1651	African green monkey kidney cells
CHEMICALS			
1-Bromooctane	Sigma-Aldrich	152951	Alkyl-halide
Allylbromide	Sigma-Aldrich	337528	Alkyl-halide
Benzylbromide	Sigma-Aldrich	B17905	Alkyl-halide
Cisplatin	Cayman Chemical	13119	Cytotoxicity control
Dichloromethane (DCM)	Sigma-Aldrich	270997	Solvent
Dimethylformamide (DMF)	Sigma-Aldrich	227056	Solvent
Dimethylsulfoxide (DMSO)	Sigma-Aldrich	276855	Solvent
DMEM, high glucose, with L-	Genesee Scientific	25-500	Cell culture media
FBS (Fetal bovine serum)	Sigma-Aldrich	F4135	Cell culture media
Furfurylamine	Acros Organics	119800050	reagent
Iodomethane	Sigma-Aldrich	289566	Alkyl-halide
Methanol	Sigma-Aldrich	34860	Solvent
MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	EMD Millipore	Calbiochem 475989-1GM	Reagent
Phosphate-buffered Saline (PBS)	Genesee Scientific	25-507	Cell culture media
REM Resin	Nova Biochem	8551010005	Polymer support; 0.500 mmol/g loading
trans -β-nitrostyrene	Sigma-Aldrich	N26806	Nitro-olefin reagent
Toluene	Sigma-Aldrich	244511	Solvent
Triethylamine (TEA)	Sigma-Aldrich	T0886	Reagent for beta-elimination
Trimethylsilyl chloride (TMSCl)	Sigma-Aldrich	386529	Reagent; CAUTION - highly volatile; creates HCl gas
GLASSWARE/INSTRUMENTATION			
25 mL solid-phase reaction vessel	Chemglass	CG-1861-02	Glassware with filter
96 Well plate reader	Promega (Turner Biosystems)	9310-011	Instrument
AVANCE III NMR Spectrometer	Bruker	N/A	Instrument; 300 MHz; Solvents: $CDCl_3$ and CD_3OH
Thermo Scientific Nicole iS5	Thermo Scientific	IQLAADGAAGFA	Instrument
Wrist-Action Shaker	Burrell Scientific	757950819	Instrument

AZUSA PACIFIC UNIVERSITY

December 2nd, 2020

Professor Benjamin Werth
Sr. Science Editor – Chemistry/Biochemistry, JoVE

Dear Dr. Werth,

Pease find attached our **REVISED** manuscript JoVE61950 titled "Functionalized Spirocyclic Heterocycle Synthesis and Cytotoxicity Assay," which includes the tracked changes (additions/deletions) within the manuscript to address the issues raised by the editor and the reviewers. Detailed specific changes are in the REBUTTAL document. As you can see, we have addressed all of the issues raised by the editor and both reviewers.

Comments from both reviewers were encouraging. Indeed, this manuscript employs the classic medicinal chemistry approach via combinatorial chemistry and MTT bioassay. Publishing detailed protocols in a video format of this drug discovery process would be invaluable to the scientific community. In light of this, we believe the manuscript is ready for publication and request that you accept this revised manuscript without further review. Thank you for your time and assistance.

Sincerely,

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Rebuttal Document

Please find below our rebuttal that addresses the comments from the editorial and the reviewers. Our rebuttal/address are in **BLUE**, editorial's comments in **BLACK**, and the reviewer's in **RED**.

Editorial comments:

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use (e.g., MTT, REM resin)**

Proof reading for spelling or grammar issues were conducted and all abbreviations were defined at first use (line #59 for REM, line #63-64 for MTT, line #109 for DMF, line #118 for DCM, line #125 for TEA, line #128 for TMSCl, and line #189 for DMSO.

2. **Please provide at least 6 keywords or phrases (max. 12).**

We included "cell viability and colorimetric assay" in lines 22-23.

3. **For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.**

The reference numbering has been changed to reflect the suggested format in lines #47, 48, 58, 64, 81, 238, 240, and 293.

4. **Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 61-67 (Anti-cancer drugs...purple formazan)**

Previous wordings "Anti-cancer drugs…purple formazan" in lines 61-67 were removed and replaced with "To determine the cytotoxicity…the purple formazan compound (Figure 2)" as seen in lines #63-69.

5. **Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**

We have added more specific details on the "how" in lines 188-190, 206-208, and 220-225.

6. **2.5: Which cells are you plating for the MTT assay? Why did you choose those cells?**

For protocol section 2.5 (line #195), COS-7 cells were identified. In addition, the rationale for the use of these cells were articulated (lines #198-202).

7. **2.12: what do you do with the absorbances at 570 and 690 nm? How do you calculate relative cell viability from these absorbance values for the various samples?**

For protocol section 2.12 (line #220), absorbance at 600 nm was measured, not 570 and 690 nm. Rationale for this was provided in lines #220-222.

8. **Although JoVE is a methods journal and publishes methods and techniques that are used as gold standards, the application of that technique or some modification has to be highlighted so that the readers and viewers can appreciate the usefulness of the method. Although you are not describing the synthesis, it would be helpful to understand what is important/different about the synthesis and characterization of these molecules, what would be points to note/take care in the synthesis and the viability measurement.**

The synthesis, though published from our previous JoVE manuscript was updated and provided in lines #81-179.

9. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: (a) Critical steps within the protocol, (b) Any modifications and troubleshooting of the technique, (c) Any limitations of the technique, (d) The significance with respect to existing methods and (e) Any future applications of the technique

For the Discussion, we made the revisions as requested in lines #292-341.

10. **Please ensure that the references appear as the following: [Last name, F.I., Last Name, F.I., Last Name, F.I. Article Title. Source. Volume (Issue), First Page–Last Page (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate any of the journal names.**

Lines #355-387 reflected the requested reference format changes.

11. **Please sort the Materials Table alphabetically by the name of the material.**

The Materials Table has been updated and items listed alphabetically by the name of the material.

Reviewer #1:

Manuscript Summary: **The proposed manuscript illustrate the famous approaches to the synthesis of MedChem relevant compounds on solid support as well as its in vitro cytotoxicity biological validation (as potential anti-cancer agents) using MTT assay. Both these methodologies are very famous in medicinal chemistry communities. Despite the popularity the real experience of the methodology using generally has industrial chemists/biologist or the academician groups involved into the projects as outsourcing partners. Therefore the publishing of the detailed protocols with the corresponding video is very interesting and useful for vide scientific community of the chemist and biologist. It also could be very efficient for the education of the students using the proposed publication in video format. Especially the demonstration of the combination of the one site solid phase synthesis - cytotoxicity check without specialized expensive equipment is really impressive for the students as well as academician chemist not deeply involved in MedChem projects.**

Comments from Reviewer #1 were encouraging. Indeed, this manuscript employs the classic medicinal chemistry approach via combinatorial chemistry and MTT bioassay. Publishing detailed protocols in a video format of this drug discovery process would be valuable to the scientific community.

Major Concerns: **The major drawbacks of the manuscript are partial repetition of the previous publication as well as absence of the real demonstration of the importance of the methodology for the medicinal chemistry. It is not so illustrative the testing of the only a few compounds using plate technique. Moreover the authors claims "Overall, the results for this assay are intended to be preliminary, as only a small number of molecules were tested. An exhaustive test with a battery of molecules is planned and a full manuscript will be forthcoming". Therefore the proposed work is seems as repeating of the previous (published in video format) with preliminary testing of the forth coming.**

As mentioned by this reviewer, only a couple of compounds were tested. Because of the recent pandemic, accessing resources has been challenging. Thus, the synthesis and analysis have been limited. However, we feel that the bioassay methodology of these spirocyclic heterocycles are complete and the initial results are encouraging. Thus we feel that these warrant publication in JoVE.

Minor Concerns: **For the better illustration of the solid support synthesis control the referring of the real picture (instead of the significant IR signal wavenumbers referring) of the IR spectra prefers (not so frequent examples accessible in the literature). Also despite a few compounds tested in MTT assay (but in different concentration) the plate layout for 96-well array with corresponding resulting plate scatter plot, % inhibition needed for the method illustration.**

Reviewer #1 suggested that we include (1) the IR spectra instead of merely providing the stretching frequency values and (2) the plate layout for the 96-well

array. We have included both of these. Revised Figure #3 (line #281-284) depicted the stacked IR spectra to monitor the progress of the synthesis. This figure would be beneficial for the audience planning similar solid phase organic synthesis. Table #1 (line #276-279) depicts the 96-well plate layout. This would also be valuable to the audience when performing similar MTT assay.

Some minor corrections of the manuscript needed like:

- **Add the reagents using for the solid synthesis into the synthetic scheme.**

Figure #1 caption (lines #270-271) includes the suggested reagents for each synthetic step.

- **Correct the reference like: "Linked Parallel Synthesis and MTT Bioassay Screening of Substituted Chalcones" instead "MTT bioassay screening of substituted chalcones" etc.**

Reference title in line #386 has been corrected.

Reviewer #2:
Manuscript Summary: The authors have described the synthesis (previously reported) and cytotoxicity assay of certain spirocyclic heterocycles. The synthesis involves an efficient solid phase methodology which is described very clearly. The cytotoxicity determination is done by the well-known MTT assay and the authors have mentioned in the abstract that they have a "refined" method for doing the assay. However the extent of "refinement" introduced has to be more clearly mentioned. If this point is addressed this manuscript will be highly useful for the research community.

Major Concerns: The modified MTT assay should be discussed with more clarity in a way that the difference from the existing assay is brought out explicitly. Otherwise the term "refined protocol" should be avoided and the authors should demonstrate the efficacy of the synthetic protocol.

Comments from Reviewer #2 were also encouraging. One remark was the word choice "refined protocol" for the MTT assay (line #38). The author is correct that we did not refined the bioassay, but adapt existing MTT assay with our spirocyclic heterocycles. So the refined language (line #38) was removed.

Minor Concerns: Will the synthesis method be applicable for amines derived from pyrrole-2-carbaldehyde as well? In that case, spirocyclic pyrrolidines will be generated which will be also be potential anticancer compounds.

Reviewer #2 saw the potential of our synthetic methodology to make spirocyclic pyrrolidines. We are excited about this possibilities and have included this for our future work (line #339-341).

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Title of Article: **Functionalized Spirocyclic Heterocycle Synthesis and Cytotoxicity Assay**

Author(s): Amelia N. Gray, Breeana M. Ramirez, Selom K. Mawugbe, Jordan F. Mar, Yun-Lan C. Wong, and Kevin S. Huang

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