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TITLE:

NMR-Based Activity Assays for Determining Compound Inhibition, IC_{50} Values, Artifactual Activity, and Whole-Cell Activity of Nucleoside Ribohydrolases

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SUMMARY:

NMR-based activity assays have been developed to identify and characterize inhibitors of two nucleoside ribohydrolase enzymes. Protocols are provided for initial compound assays at 500 μM and 250 μM, dose-response assays for determining IC_{50} values, detergent counter screen assays, jump-dilution counter screen assays, and assays in *E. coli* whole cells.

ABSTRACT:

NMR spectroscopy is often used for the identification and characterization of enzyme inhibitors in drug discovery, particularly in the context of fragment screening. NMR-based activity assays are ideally suited to work at the higher concentrations of test compounds required to detect these weaker inhibitors. The dynamic range and chemical shift dispersion in an NMR experiment

can easily resolve resonances from substrate, product, and test compounds. This contrasts with spectrophotometric assays, in which read-out interference problems often arise from compounds with overlapping UV-vis absorption profiles. In addition, since they lack reporter enzymes, the single-enzyme NMR assays are not prone to coupled-assay false positives. This attribute makes them useful as orthogonal assays, complementing traditional high throughput screening assays and benchtop triage assays. Detailed protocols are provided for initial compound assays at 500 μM and 250 μM, dose-response assays for determining IC_{50} values, detergent counter screen assays, jump-dilution counter screen assays, and assays in *E. coli* whole cells. The methods are demonstrated using two nucleoside ribohydrolase enzymes. The use of ^{1}H NMR is shown for the purine-specific enzyme, while ^{19}F NMR is shown for the pyrimidine-specific enzyme. The protocols are generally applicable to any enzyme where substrate and product resonances can be observed and distinguished by NMR spectroscopy. To be the most useful in the context of drug discovery, the final concentration of substrate should be no more than 2–3x its K_m value. The choice of NMR experiment depends on the enzyme reaction and substrates available as well as available NMR instrumentation.

INTRODUCTION:

Nuclear magnetic resonance (NMR) spectroscopy is well-established for characterizing and monitoring enzyme reactions[1,2]. Differences in chemical shifts and coupling patterns are used to distinguish substrate and product resonances, and relative resonance intensities are used to quantify the percent of reaction. Both the consumption of substrate and the creation of product are directly observed in the NMR spectrum. This contrasts with spectrophotometry or fluorescence spectroscopy, in which the reaction time course is indicated by a change in absorbance attributable to some chemical species being consumed or created. Just as with the other methods, NMR can be used to study enzyme reactions as a function of temperature, pH, or other solution conditions, and the effects of inhibitors can be determined.

More recently, NMR-based enzyme activity assays have been demonstrated for fragment screening[3,4]. NMR-based assays are ideally suited to work at the higher concentrations of test compounds (often as high as 1 mM) required to detect these weaker inhibitors. The dynamic range and chemical shift dispersion in the NMR experiment can easily resolve resonances from substrate, product, and test compounds. This compares favorably to spectrophotometric assays where read-out interference problems often arise from compounds with overlapping UV-vis absorption profiles. In addition, since they lack reporter enzymes, the single-enzyme NMR assays are not prone to coupled-assay false positives. This advantage makes them useful as orthogonal assays, complementing traditional high throughput screening assays and benchtop triage assays[5].

In our research laboratory, NMR-based activity assays are used to identify and evaluate inhibitors of *Trichomonas vaginalis* nucleoside ribohydrolases. The *T. vaginalis* parasite causes the most prevalent non-viral sexually transmitted disease[6]. Increasing resistance to existing therapies[7] is driving the need for novel, mechanism-based treatments, with essential nucleoside salvage pathway enzymes representing prime targets[8]. NMR-based activity assays have been developed for both pyrimidine- and purine-specific enzymes, uridine nucleoside ribohydrolase (UNH)[9], and

adenosine/guanosine preferring nucleoside ribohydrolase (AGNH)[10]. The reactions catalyzed by these two enzymes are shown in **Figure 1**. The NMR assays are being used to screen fragment libraries for chemical starting points, determine IC_{50} values, and weed out aggregation-based or covalent binding inhibitors[11]. The same assays are also being translated to assess enzyme activity in whole cells[12].

Detailed protocols are provided for initial compound assays at 500 μM and 250 μM, dose-response assays for determining IC_{50} values, detergent counter screen assays, jump-dilution counter screen assays, and assays in *E. coli* whole cells. The protocols are generally applicable to any enzyme in which substrate and product resonances can be observed and distinguished by NMR spectroscopy. Three assumptions have been made for simplicity. First, the substrate is not specified. For NMR-based activity assays to be useful, the final concentration of substrate should be no more than 2–3x the K_m value[4]. In the examples shown, the final concentrations of adenosine and 5-fluorouridine are 100 μM (K_m = 54 μM) and 50 μM (K_m = 15 μM), respectively. In the protocols, achieving these concentrations corresponds to 12 μL of 5 mM adenosine or 12 μL of 2.5 mM 5-fluorouridine.

Second, the amount of enzyme provided for in the protocols, 5 μL, was chosen to correspond to the amount required to result in approximately 75% conversion of substrate to product in 30 min. This quantity typically represents a large dilution from a purified enzyme stock, and the dilution must be determined in advance for each enzyme. Purified AGNH and UNH enzyme stock solutions are stored at -80 °C in aliquots that provide enough enzyme for several thousand reactions. Thus, the dilution factor ideally only needs to be determined or validated every few months. Third, the specific 1D NMR experiment is not specified. In the representative results, ^{1}H NMR is shown for AGNH[10] and ^{19}F NMR is shown for UNH[9], with the NMR experiment described in the corresponding references. The choice of NMR experiment depends on the enzyme reaction and substrates available as well as available NMR instrumentation. Finally, it should be pointed out that the experimental approach described does not adhere to the strict requirements of quantitative NMR (qNMR)[13,14]. In the protocol, a percent reaction is determined using the relative changes in intensity of the same resonance in each spectrum, rather than by determining absolute concentrations. This approach eliminates the need for data acquisition and processing modifications as well as internal or external standards, which are required for qNMR.

PROTOCOLS:

1. Initial test compound assays at 500 μM and 250 μM

1.1. Prepare substrate and test compound for reactions.

1.1.1. Prepare stock solutions of substrate (adenosine or 5-fluorouridine) in water and 50 mM test compound in deuterated dimethyl sulfoxide (DMSO). Refer to the introduction section for concentrations of substrate solution to use.

1.1.2. Add 12 μL of substrate (adenosine or 5-fluorouridine) to each of four 1.5 mL microfuge tubes, 1–4.

1.1.3. Add 6 μL of deuterated DMSO to tubes 1 (0 min control) and 4 (30 min control). Add 6 μL of test compound to tube 2. Add 3 μL of test compound and 3 μL of deuterated DMSO to tube 3.

1.2. Prepare sufficient reaction stock solution.

NOTE: The stock solution is for five reactions that each contain 517 μL of buffer, 60 μL of deuterium oxide, and 5 μL of enzyme solution (AGNH or UNH). Refer to the introduction section for concentrations of enzyme solution to use.

1.2.1. Add 2.59 mL of reaction buffer (50 mM potassium phosphate, 0.3 M KCl, pH 6.5) to a 15 mL conical tube. Add 300 μL of deuterium oxide to the conical tube. Add 25 μL of enzyme solution (AGNH or UNH) to the conical.

1.2.2. Gently invert the conical tube twice to mix.

1.3. Simultaneously initiate and quench the 0 min control reaction. Transfer 582 μL of the reaction stock solution to a clean microfuge tube. Add 10 μL of 1.5 M HCl to this microfuge tube. Transfer the combined 592 μL to microfuge tube 1. Aspirate and dispense the sample twice in a slow but deliberate fashion.

1.4. Initiate and run the remaining three reactions in staggered fashion. At time 0 min, transfer 582 μL of the reaction stock solution to microfuge tube 2. Aspirate and dispense the sample twice in a slow but deliberate fashion. Repeat at 30 s intervals for microfuge tubes 3 and 4. Wait 30 min.

1.5. Quench the reactions. At time 30 min, add 10 μL of 1.5 M HCl to microfuge 2. Repeat at 30 s intervals for microfuge tubes 3 and 4. Transfer 600 μL of solution from each microfuge to NMR tubes.

1.6. Acquire a 1D NMR spectrum on each sample. Process the data to ensure correct phasing and flat baselines.

1.7. Calculate the percent conversion of substrate for control spectra.

1.7.1. Overlay the spectra for 0 min and 30 min controls. Scale the substrate signal in the 0 min control to match the 30 min control. Note this percentage.

1.7.2. Calculate the percent conversion as (100 – percentage determined in step 1.7.2).

1.8. Calculate the percent conversion of substrate for reactions containing test compound.

1.8.1. Overlay spectra for the 0 min control and first reaction containing the 500 μM test compound. Scale the substrate signal in the 0 min control to match the spectrum with test compound. Note this percentage. Calculate percent conversion as (100 – percentage determined in step 1.8.2).

1.8.2. Repeat for the second reaction containing the 250 μM test compound.

1.9. Calculate the percent reaction and percent inhibition for each test compound concentration.

1.9.1. Calculate the percent reaction as (1.8.3/1.7.3) x 100.

1.9.2. Calculate the percent inhibition as (100 – percentage determined in step 1.9.1).

2. Determination of IC_{50} values

2.1. Prepare substrate and test compound for reactions.

2.1.1. Prepare stock solutions of substrate (adenosine or 5-fluorouridine) in water and 10 mM test compound in deuterated DMSO. Refer to the introduction section for concentrations of substrate solution to use.

2.2. Prepare serial dilutions of 10 mM test compound (in deuterated DMSO).

2.2.1. Add 36 μL deuterated DMSO to five 1.5 mL microfuge tubes, 1–5.

2.2.2. Add 12 μL 10 mM test compound to tube 1 and tap lightly to mix. Test compound is now 2.5 mM.

2.2.3. Transfer 12 μL from tube 1 to tube 2 and tap lightly to mix. Test compound is now 0.63 mM. Transfer 12 μL from tube 2 to tube 3 and tap lightly to mix. Test compound is now 0.16 mM. Transfer 12 μL from tube 3 to tube 4 and tap lightly to mix. Test compound is now 0.04 mM. Transfer 12 μL from tube 4 to tube 5 and tap lightly to mix. Test compound is now 0.01 mM.

2.3. Prepare 14 1.5 mL microfuge tubes for reactions.

2.3.1. Add 12 μL of substrate (adenosine or 5-fluorouridine) to each of 14 1.5 mL microfuge tubes, 1-14.

2.3.2. Add 12 μL of deuterated DMSO to tubes 1 (0 min control) and 14 (30 min control).

2.3.3. Add 12 μL of 10 mM test compound to tubes 2 and 3. Add 12 μL of 2.5 mM test compound to tubes 4 and 5. Add 12 μL of 0.63 mM test compound to tubes 6 and 7. Add 12 μL of 0.16 mM test compound to tubes 8 and 9. Add 12 μL of 0.04 mM test compound to tubes 10 and 11. Add 12 μL of 0.01 mM test compound to tubes 12 and 13.

2.4. Prepare sufficient reaction stock solution to run 15 reactions that contain 511 μL of buffer, 60 μL of deuterium oxide, and 5 μL of enzyme solution (AGNH or UNH) each. Refer to the introduction section for concentrations of enzyme solution to use.

2.4.1. Add 7.67 mL of reaction buffer (50 mM potassium phosphate, 0.3 M KCl, pH = 6.5) to a 15 mL conical tube. Add 900 μL of deuterium oxide to the conical tube. Add 75 μL of enzyme solution (AGNH or UNH) to the conical.

2.4.2. Gently invert the conical tube 2x to mix.

2.5. Follow the steps outlined in steps 1.3–1.9 to (using 576 μL of reaction stock solution) to determine percent reaction for each test compound concentration.

2.6. Calculate IC_{50} value using GraphPad Prism.

2.6.1. Create a data table correlating the log of reaction test compound concentrations (200 μM, 50 μM, 12.5 μM, 3.13 μM, 0.78 μM, 0.20 μM) with percent reaction (two values each).

2.6.2. Use a nonlinear curve fit to determine the IC_{50} value and standard error.

3. Detergent Counter Screen Assays at 100 μM and 50 μM

3.1. Prepare substrate and test compound for reactions.

3.1.1 Prepare stock solutions of substrate (adenosine or 5-fluorouridine) in water and 10 mM test compound in deuterated DMSO. Refer to the introduction section for concentrations of substrate solution to use.

3.1.2. Add 12 μL of substrate (adenosine or 5-fluorouridine) to each of eight 1.5 mL microfuge tubes, 1–8.

3.1.3. Add 6 μL of deuterated DMSO to tubes 1 and 5 (0 min controls) and 4 and 8 (30 min controls). Add 6 μL of test compound to tubes 2 and 6. Add 3 μL of test compound and 3 μL of deuterated DMSO to tubes 3 and 7.

3.2. Prepare sufficient reaction stock solution WITHOUT detergent to run five reactions that contain 517 μL of buffer, 60 μL of deuterium oxide, and 5 μL of enzyme solution (AGNH or UNH) each. Refer to the introduction section for concentrations of enzyme solution to use.

3.2.1. Add 2.59 mL of reaction buffer (50 mM potassium phosphate, 0.3 M KCl, pH = 6.5) to a 15 mL conical tube. Add 300 μL of deuterium oxide to the conical tube. Add 25 μL of enzyme solution (AGNH or UNH) to the conical.

3.2.2. Gently invert the conical tube 2x to mix.

3.3. Prepare sufficient reaction stock solution WITH 0.01% v/v Triton X-100 detergent to run five reactions that contain 517 μL of buffer, 60 μL of deuterium oxide, and 5 μL of enzyme solution (AGNH or UNH) each.

3.3.1. Add 2 μL of Triton X-100 detergent to 20 mL of reaction buffer (50 mM potassium phosphate, 0.3 M KCl, pH = 6.5). Detergent solutions must be used within 1 day.

3.3.2. Add 2.59 mL of reaction buffer containing 0.01% Triton X-100 detergent to a 15 mL conical tube. Add 300 μL of deuterium oxide to the conical tube. Add 25 μL of enzyme solution (AGNH or UNH) to the conical.

3.3.3. Gently invert the conical tube 2x to mix.

3.4. For tubes 1–4, using the reaction stock solution WITHOUT detergent, follow the steps outlined in steps 1.3–1.9 to determine the percent inhibition for each test compound concentration.

3.5. For tubes 5–8, using the reaction stock solution WITH 0.01% v/v Triton X-100 detergent, follow the steps outlined in steps 1.3–1.9 to determine the percent inhibition for each test compound concentration.

4. Jump-dilution counter screen assays

4.1. Prepare the substrate and test compounds for reactions

4.1.1 Prepare stock solutions of substrate (adenosine or 5-fluorouridine) in water and 10 mM test compound in deuterated DMSO. Refer to the introduction section for concentrations of substrate solution to use.

4.1.2. Add 53.8 μL of reaction buffer (50 mM potassium phosphate, 0.3 M KCl, pH = 6.5) to two 1.5 mL microfuge tubes (each), 1 and 2. Add 5 μL of enzyme (AGNH or UNH) to tubes 1 and 2.

4.1.3 Add 511 μL of reaction buffer to two 1.5 mL microfuge tubes, 3 and 4 (each). Add 60 μL of deuterium oxide to tubes 3 and 4. Add 5 μL of enzyme (AGNH or UNH) to tubes 3 and 4.

4.1.4. Add 1.2 μL of deuterated DMSO to tube 1 (30 min control). Add 1.2 μL of test compound to tube 2. Add 12 μL of deuterated DMSO to tube 3 (30 min control). Add 12 μL of test compound to tube 4. Incubate for 30 min.

4.2. Prepare two 1.5 mL microfuge tubes with dilution solution.

4.2.1. Add 468 μL of reaction buffer to each of two 1.5 mL microfuge tubes, 5 and 6. Add 60 μL of deuterium oxide to each tube (5 and 6).

4.3. Prepare four 1.5 mL microfuge tubes for the reactions.

4.3.1. Add 12 μL of substrate (adenosine or 5-fluorouridine) to each of four 1.5 mL microfuge tubes, 7–10.

4.4. Perform jump-dilutions and initiate the reactions.

4.4.1. Transfer 528 μL of solution from tube 5 to tube 1. Aspirate and dispense the sample twice in a slow but deliberate fashion. Transfer 528 μL of solution from tube 6 to tube 2. Aspirate and dispense the sample twice in a slow but deliberate fashion.

4.4.2. At time 0 min, transfer 588 μL of solution from tube 1 to tube 7. Aspirate and dispense the sample 2x in a slow but deliberate fashion. At 30 s intervals, transfer 588 μL of solution from tube 2 to tube 8, tube 3 to tube 9, and tube 4 to tube 10. Aspirate and dispense the sample twice in a slow but deliberate fashion. Wait 30 min.

4.4. For tubes 7–10, follow the steps outlined in steps 1.5–1.9 to determine the percent inhibition for each test compound concentration.

5. Compound assays in *E. coli* whole cells

5.1. Prepare 10 mL overnight culture of *E. coli* on day preceding experiments.

5.2. Prepare *E. coli* cells for NMR experiments.

5.2.1. Centrifuge the cells in 1 mL aliquots for 10 min at 15,000 x *g*.

5.2.2. Discard the supernatant and resuspend each aliquot of cells in 1 mL of reaction buffer (50 mM potassium phosphate, 0.3 M KCl, pH = 6.5) by vortexing.

5.3. Follow sections 1 or 2 for the desired assay with the following changes:

5.3.1. Substitute 280 μL of buffer in the reaction stock solution with 280 μL of resuspended cells.

5.3.2. Substitute 5 μL of enzyme (AGNH or UNH) in the reaction stock solution with 5 μL of buffer.

REPRESENTATIVE RESULTS:

Figure 2 shows the results for testing two compounds against AGNH using ^{1}H NMR following section 1. The enzyme reaction is most easily observed and quantified by the disappearance of adenosine singlet and doublet resonances at 8.48 ppm and 6.09 ppm, respectively, and the appearance of an adenine singlet resonance at 8.33 ppm as observed in the 30 min control spectrum. In the presence of 500 μM compound 1, no product is formed as evidenced by the lack of an adenine resonance at 8.33 ppm. In the presence of 250 μM compound 1, about 10% of the substrate has been converted to product. By contrast, neither concentration of compound 2 inhibits the enzyme as evidenced by the substrate and product resonances resembling those in the 30 min control spectrum. This data identifies compound 1 as a good AGNH inhibitor. Note that resonances arising from compound 1 (7.70–8.00 and 8.50–8.60 ppm) and compound 2 (7.40–7.80 ppm) are also observed. The same substrate and product resonances are used to monitor the reactions shown in **Figure 4, Figure 6, Figure 8,** and **Figure 10**. **Figure 3** shows the results for testing two compounds against UNH using ^{19}F NMR following section 1. The enzyme reaction is easily observed and quantified by the disappearance of the uridine resonance at -165.8 ppm and the appearance of a uracil resonance at -169.2 ppm as observed in the 30 min control spectrum. For this enzyme, compound 2 completely inhibits the reaction at both concentrations whereas compound 1 has no effect. This data identifies compound 2 as a good UNH inhibitor. The same substrate and product resonances are used to monitor the reactions shown in **Figure 5, Figure 7, Figure 9,** and **Figure 11**.

Figure 4 shows the dose-response NMR data and resulting IC_{50} curve obtained for a compound with AGNH activity using ^{1}H NMR following section 2. NMR data is shown for only one of the duplicate trials. Note that resonances arising from the tested compound (6.90–7.40 ppm) do not interfere with the substrate or product resonances. The IC_{50} curve was fit using data from both trials and resulted in a value of 12.3 ± 5.0 μM. This result is consistent with the NMR data in that significant loss of substrate signal is not observed until the compound concentration is reduced to 12.5 μM. **Figure 5** shows the dose-response NMR data and resulting IC_{50} curve obtained for a compound with UNH activity using ^{19}F NMR following section 2. NMR data is shown for only one of the duplicate trials. The IC_{50} curve was fit using data from both trials and resulted in a value of 16.7 ± 10.4 μM. This value is consistent with the NMR data in that significant loss of substrate signal is not observed until the compound concentration is reduced to 12.5 μM.

Figure 6 shows the results for testing a compound at two concentrations against AGNH in the absence and presence of 0.01% Triton X-100 detergent using ^{1}H NMR following section 3. Only minimal differences are observed in the intensities of the substrate and product signals using the two conditions, indicating that the observed enzyme inhibition is not an artifact of compound aggregation. Note that resonances arising from the tested compound (7.10–7.70 ppm) and Triton X-100 (6.90 and 7.40 ppm) do not interfere with the substrate or product resonances. **Figure 7**

shows the results for testing a compound at two concentrations against UNH in the absence and presence of 0.01% Triton X-100 detergent using ^{19}F NMR following section 3. Only minimal differences are observed in the intensities of the substrate and product signals using the two conditions, indicating that the observed enzyme inhibition is not an artifact of compound aggregation.

Figure 8 shows the results for testing a compound in the jump-dilution assay against AGNH using ^{1}H NMR following section 4. The reduced intensity of the substrate signal in the 20 μM reaction compared to the 200 μM reaction indicates that the inhibition is reversible. The tested compound has an IC_{50} value of 21.0 μM, and its resonances are observed at 6.90–8.30 ppm. In this example, resonances from the compound interfere with those of the adenine product signal, and reaction progress is easier to monitor using the adenosine resonance at 6.09 ppm. **Figure 9** shows the results for testing a compound in the jump-dilution assay against UNH using ^{19}F NMR following section 4. The increased intensity of the product signal at -169.2 ppm in the 20 μM reaction compared to the 200 μM reaction indicates that the inhibition is reversible. The tested compound has an IC_{50} value of 16.7 μM.

Figure 10 shows the utility of the method for performing assays in whole cells using ^{1}H NMR following section 5. The adenosine substrate signals are almost completely gone after 30 min in the presence of whole cells, indicating hydrolysis of the substrate. By contrast, the substrate remains unchanged after 30 min in the presence of cell growth media supernatant, indicating that the hydrolysis reaction is cell dependent. Note the presence of many background signals in the supernatant spectra, including intense triplet signals from NH_4^+ ions at 6.90–7.15 ppm that are also present to a smaller degree in the whole cell spectra. **Figure 11** shows the utility of the method for performing assays in whole cells using ^{19}F NMR following section 5. The uridine substrate signal is completely gone after 60 min in the presence of whole cells, indicating hydrolysis of the substrate. By contrast, the substrate remains unchanged after 60 min in the presence of cell growth media supernatant, indicating that the hydrolysis reaction is cell dependent.

FIGURE AND TABLE LEGENDS:

Figure 1: Reactions catalyzed by UNH (top) and AGNH (bottom). Note that UNH will catalyze the hydrolysis of both uridine and 5-fluorouridine (shown).

Figure 2: Representative initial compound assays at 500 μM and 250 μM against AGNH using ^{1}H NMR. Regions of the ^{1}H NMR reaction spectra for two compounds, each at 500 μM and 250 μM, along with 0 min and 30 min control spectra. The 0 min control spectrum contains adenosine substrate resonances at 6.09, 8.38, and 8.48 ppm. The 30 min control spectrum contains a new adenine product resonance at 8.33 ppm. Test spectra contain additional resonances arising from the compound tested. ^{1}H chemical shifts were referenced to external trimethylsilylpropionic acid at 0.0 ppm.

Figure 3: Representative initial compound assays at 500 μM and 250 μM against UNH using ^{19}F NMR. Regions of the ^{19}F NMR reaction spectra for two compounds, each at 500 μM and 250 μM, along with 0 min and 30 min control spectra. The 0 min control spectrum contains a uridine substrate resonance at -165.8 ppm. The 30 min control spectrum contains a new uracil product resonance at -169.2 ppm. ^{19}F chemical shifts were referenced to external 50 μM trifluoroethanol at -76.7 ppm.

Figure 4: Representative dose-response NMR data and resulting IC_{50} curve obtained for a compound with AGNH activity using ^{1}H NMR. Regions of the ^{1}H NMR reaction spectra for variable concentrations of a compound (200–0.20 μM) along with 0 min and 30 min control spectra. Resonances from 6.90–7.40 ppm arise from the tested compound. The IC_{50} curve was fit using data from NMR data sets run in duplicate.

Figure 5: Representative dose-response NMR data and resulting IC_{50} curve obtained for a compound with UNH activity using ^{19}F NMR. Regions of the ^{19}F NMR reaction spectra for variable concentrations of a compound (200–0.20 μM) along with 0 min and 30 min control spectra. The IC_{50} curve was fit using data from NMR data sets run in duplicate.

Figure 6: Representative detergent counter screen assays for a compound with AGNH activity using ^{1}H NMR. Regions of the ^{1}H NMR reaction spectra for a compound at 100 μM and 50 μM, along with 0 min and 30 min control spectra, in the presence and absence of 0.01% Triton X-100. Resonances at 7.10–7.70 ppm and 6.90 and 7.40 ppm arise from the tested compound and Triton X-100, respectively.

Figure 7: Representative detergent counter screen assays for a compound with UNH activity using ^{19}F NMR. Regions of the ^{19}F NMR reaction spectra for a compound at 100 μM and 50 μM, along with 0 min and 30 min control spectra, in the presence and absence of 0.01% Triton X-100.

Figure 8: Representative jump-dilution counter screen assays for a compound with AGNH activity using ^{1}H NMR. Regions of the ^{1}H NMR reaction spectra for a compound at 200 μM and 20 μM, along with 30 min control spectra. Enzyme was incubated for 30 min at 200 μM compound prior to the start of the reactions, with the 20 μM reaction diluted immediately before initiating the reaction. Resonances at 6.90–8.30 ppm arise from the tested compound.

Figure 9: Representative jump-dilution counter screen assays for a compound with UNH activity using ^{19}F NMR. Regions of the ^{19}F NMR reaction spectra for a compound at 200 μM and 20 μM, along with 30 min control spectra. Enzyme was incubated for 30 min at 200 μM compound prior to the start of the reactions, with the 20 μM reaction diluted immediately before initiating the reaction.

Figure 10: Representative assays in whole cells using ^{1}H NMR. Regions of the ^{1}H NMR reaction spectra for samples containing either 280 μL of *E. coli* cells resuspended in buffer (0, 15, and 30 min) or cell growth media supernatant (30 min).

Figure 11: Representative assays in whole cells using ^{19}F NMR. Regions of the ^{19}F NMR reaction spectra for samples containing either 280 μL of *E. coli* cells resuspended in buffer (0, 15, 30, and 60 min) or cell growth media supernatant (60 min).

DISCUSSION:

The protocols described are generally applicable to many enzymes, provided that the substrates and/or products have resolvable signals in the NMR spectrum. However, it is critical that the concentration of substrate is close to its K_m value and high enough to be detected in an NMR experiment within a reasonable timeframe. A substrate concentration no higher than 2–3x the K_m value is optimal for detecting competitive, noncompetitive, and uncompetitive inhibitors[4]. As demonstrated here for UNH, substrate, K_m values as low as 15 μM are suitable. The use of substrates with CH_3 or CF_3 signals, coupled with NMR data collection on cryogenic probes, can lower this threshold even further[15]. Enzymes that have substrate K_m values below 1 μM, however, are likely difficult to study using this method because of the inherent low sensitivity of the NMR experiment. In these situations, spectrophotometry or fluorescence spectroscopy are more suitable techniques.

Another limitation to the application of these methods is a suitable quenching agent. All of the protocols described here are fixed-time assays, with the reaction quenched after 30 min by the addition of HCl. For both AGNH and UNH, it had been previously determined that HCl immediately stopped the reaction, and kept it stopped for periods of weeks[9, 10]. This is important since dozens of reactions are often run simultaneously and then queued for NMR data collection over a period of several hours. It is also important to establish that non-enzymatic degradation of the substrate or product signals does not occur subsequent to quenching but prior to NMR data collection. In addition to HCl, other common ways to quench reactions are by the addition of a known nanomolar inhibitor[16] or, in the case of reactions involving adenosine triphosphate, the addition of chelating agent ethylenediaminetetraacetic acid[17].

NMR-based activity assays provide added value when used for fragment screening or orthogonal assays to validate high-throughput screening hits[4]. In contrast to binding assays, NMR-based activity assays identify or confirm compounds as actual inhibitors. The activity assays also use far less target enzyme than do binding assays. The same methods are incredibly robust for two types of counter screens carried out to validate reversible, target-specific activity and rule out artifactual assay activity[18]. Detergent and jump-dilution assays are counter-screens for colloidal aggregation[19] and irreversible inhibition[20], respectively. Compounds that pass these tests are validated starting points for medicinal chemistry and structure-guided inhibitor optimization. The NMR-based activity assays can continue to provide compound activity data as the project progresses toward nanomolar inhibitors.

Finally, the utility of these assays for monitoring reactions in whole cells is noteworthy[21]. Correlating inhibition of purified enzyme with inhibition of in-cell enzyme would provide definitive proof of the biochemical mechanism underlying the observed phenotypic effect[22]. For

the two enzymes presented here, the desired phenotypic effect is loss of viability (antitrichomonal activity). A correlation between purified enzyme inhibition, in-cell enzyme inhibition, and parasite cell death would constitute proof of the inhibitor mechanism of action.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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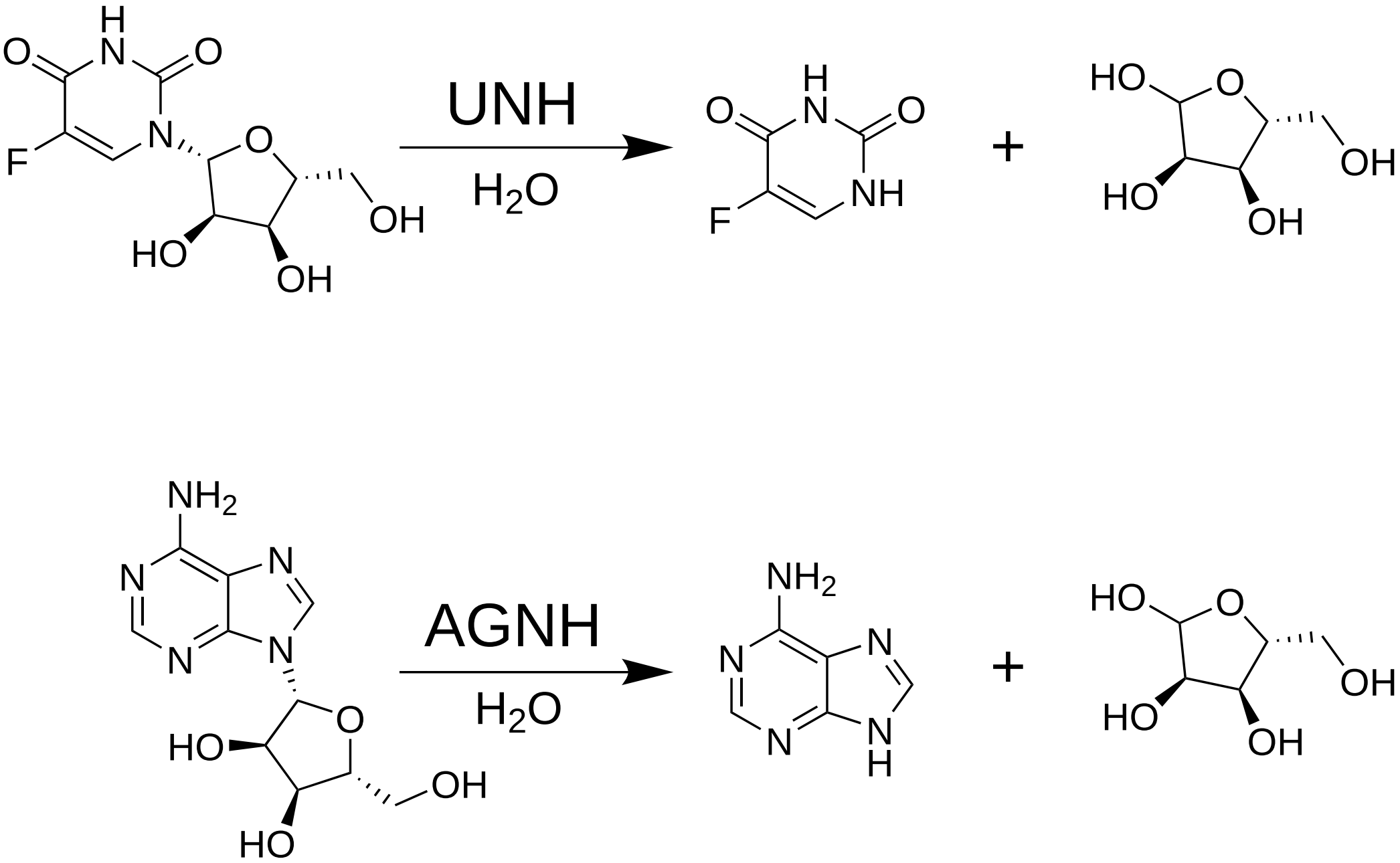

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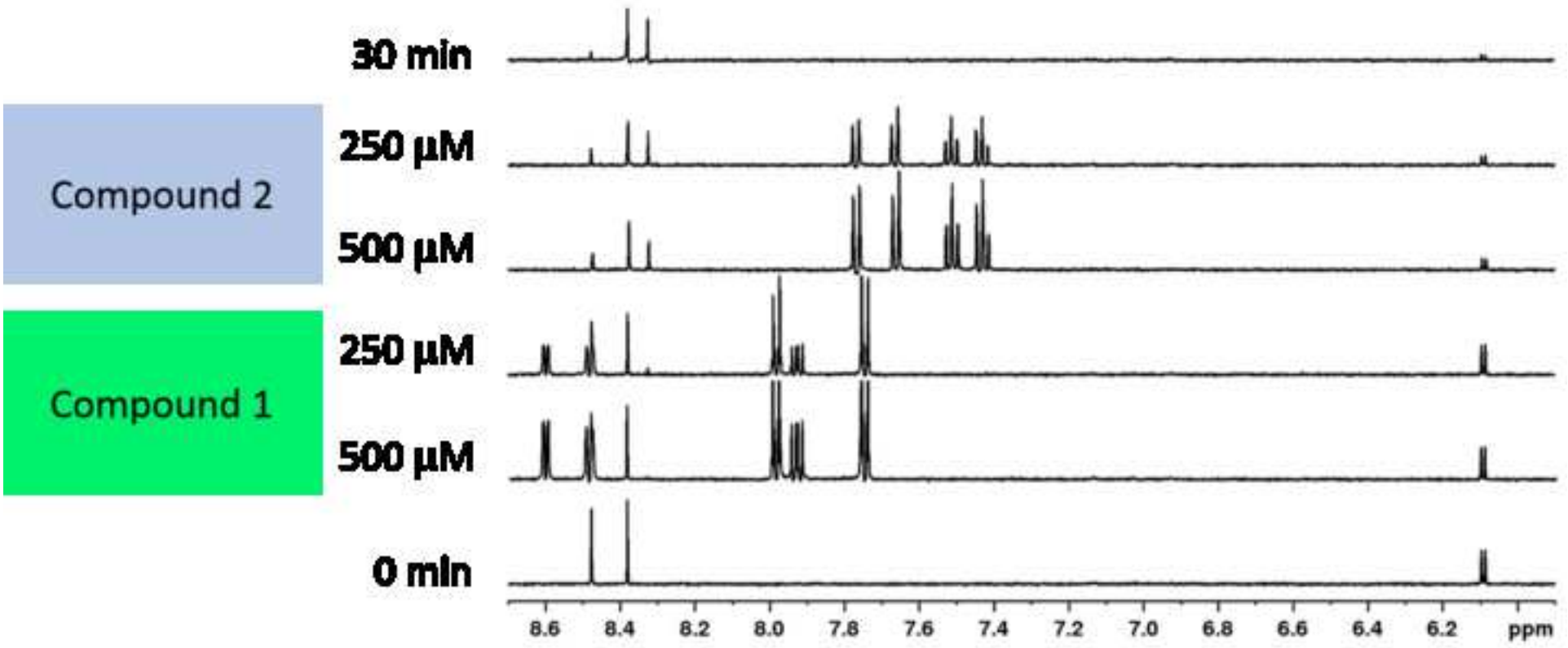

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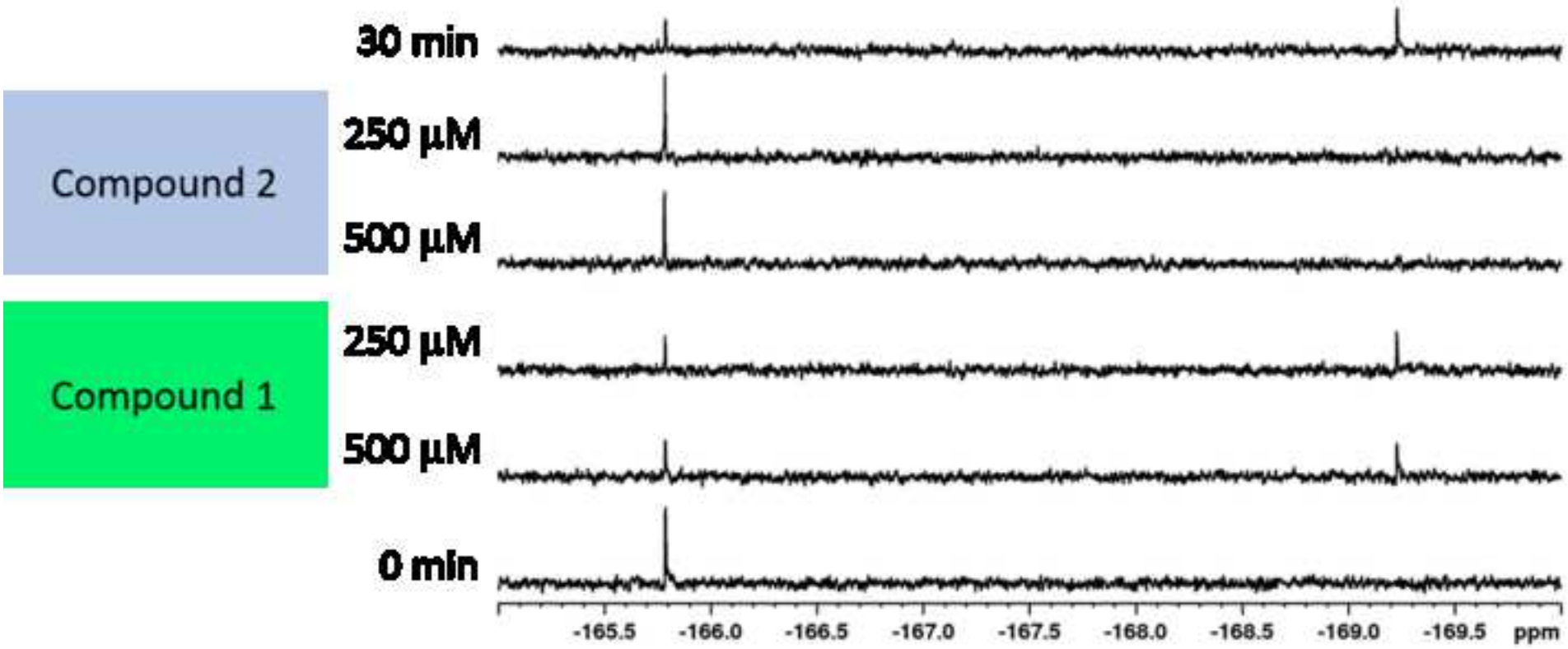

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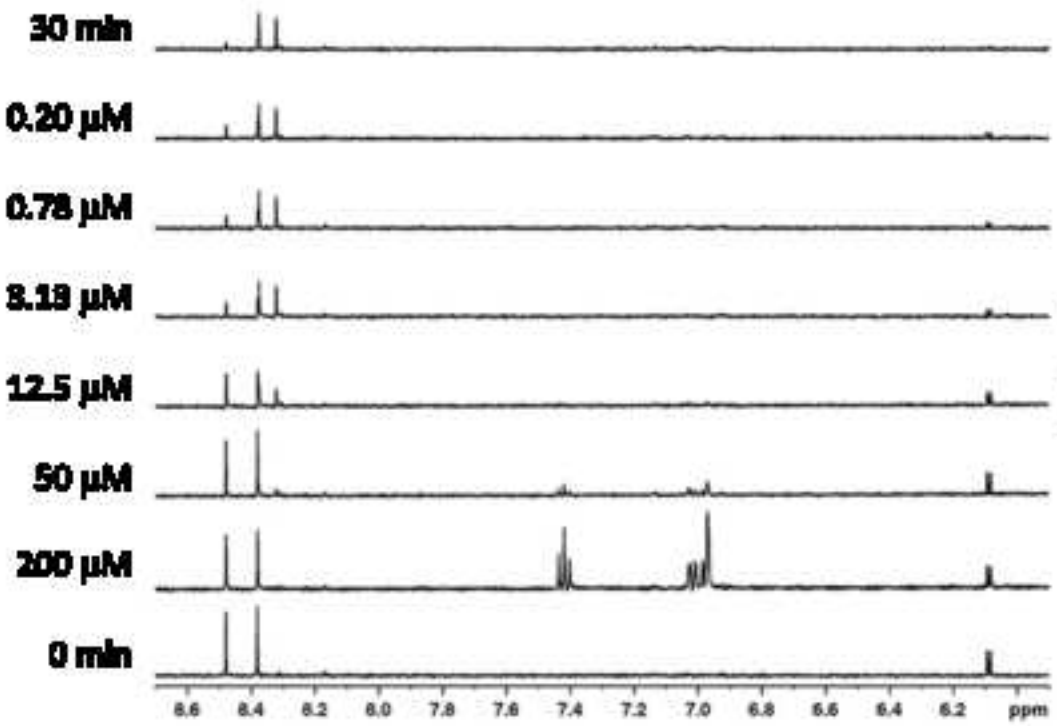

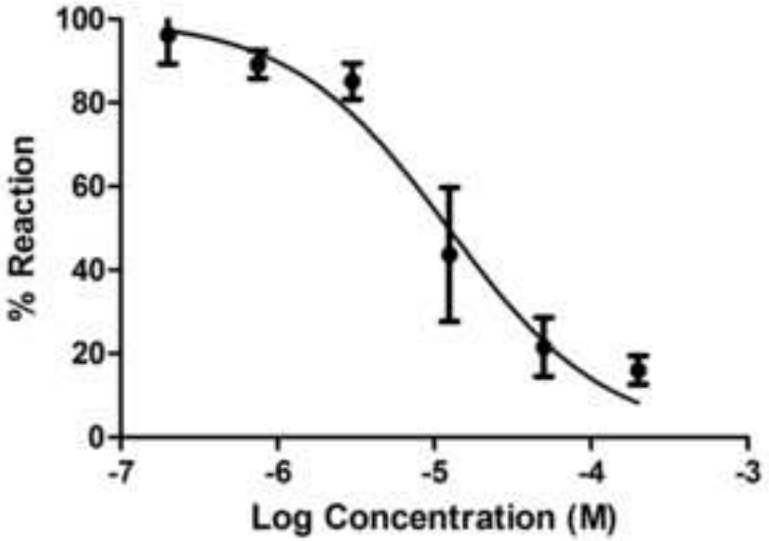

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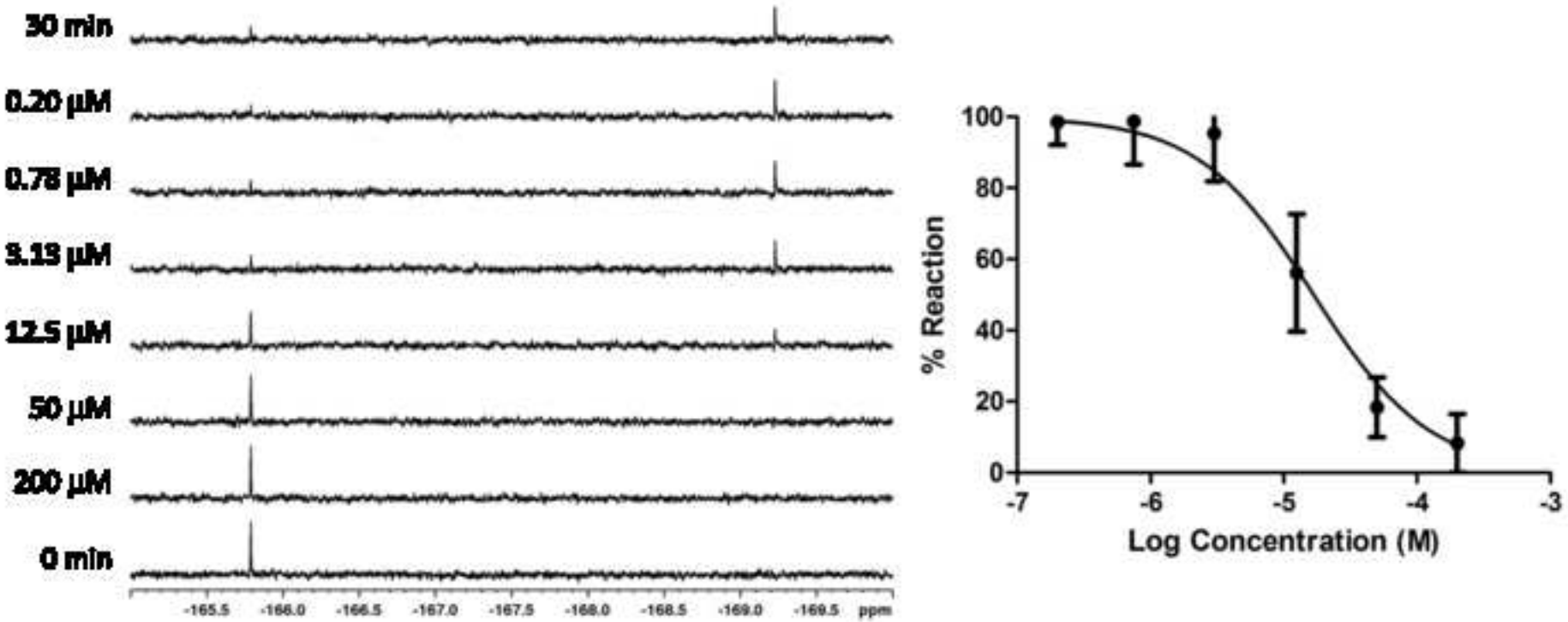

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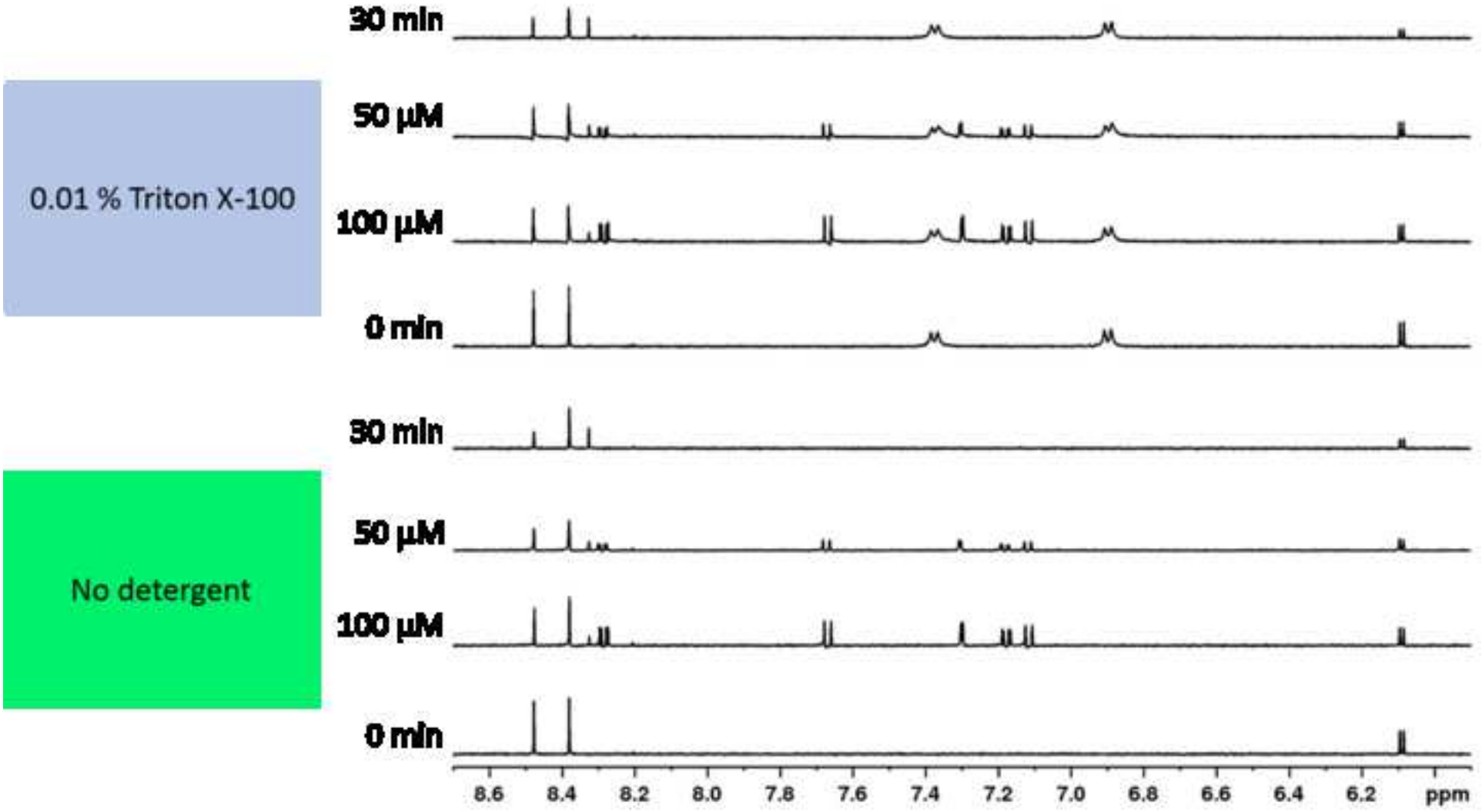

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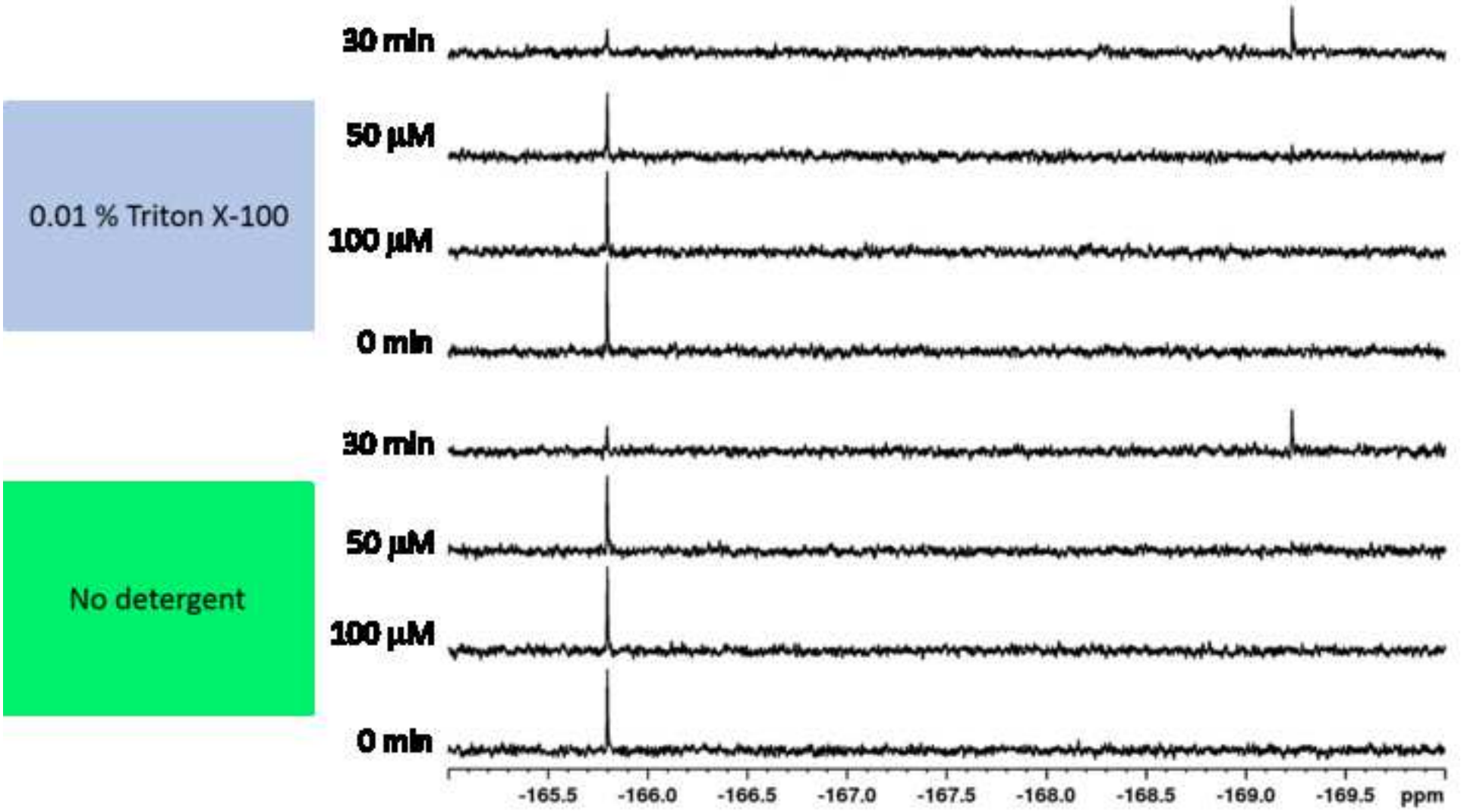

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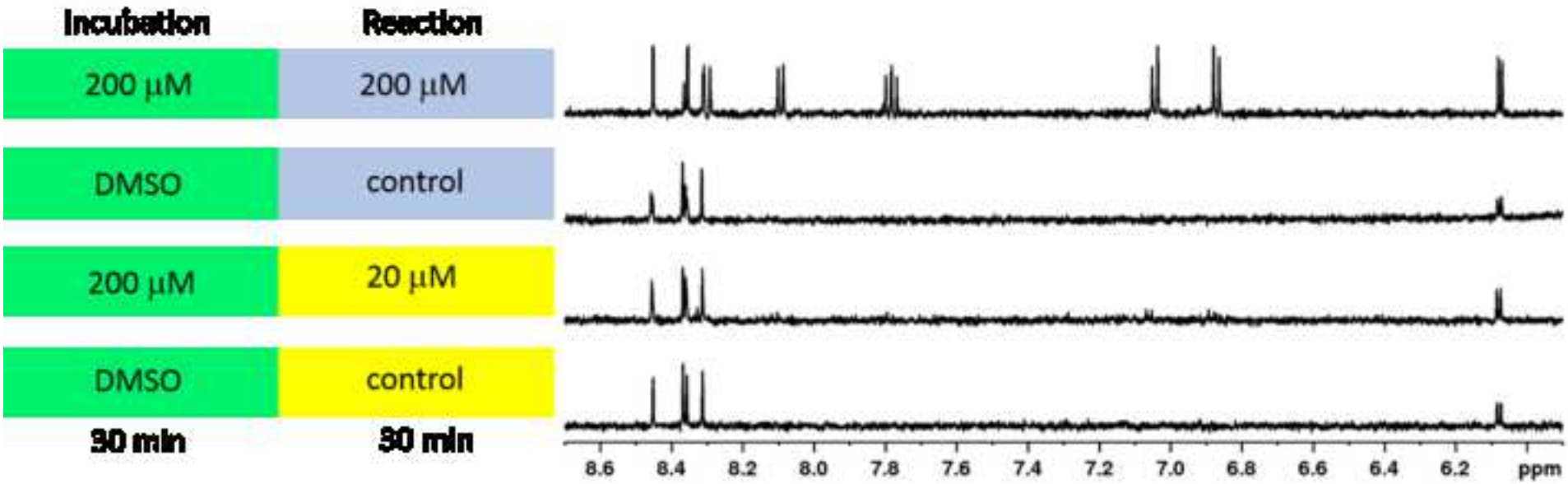

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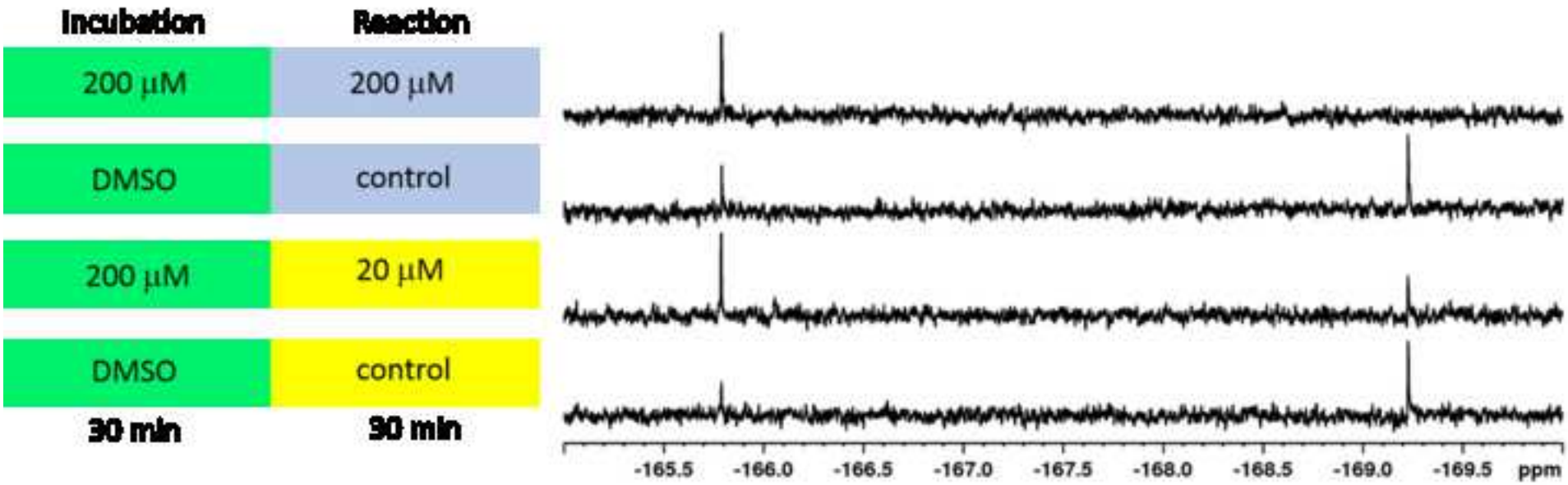

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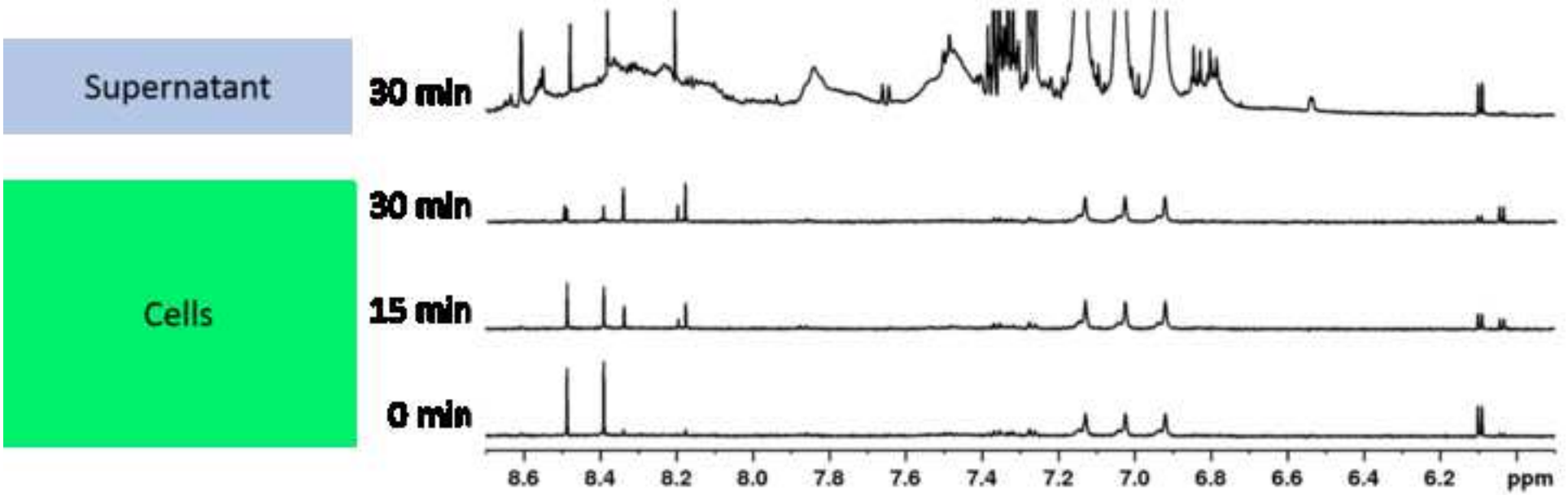

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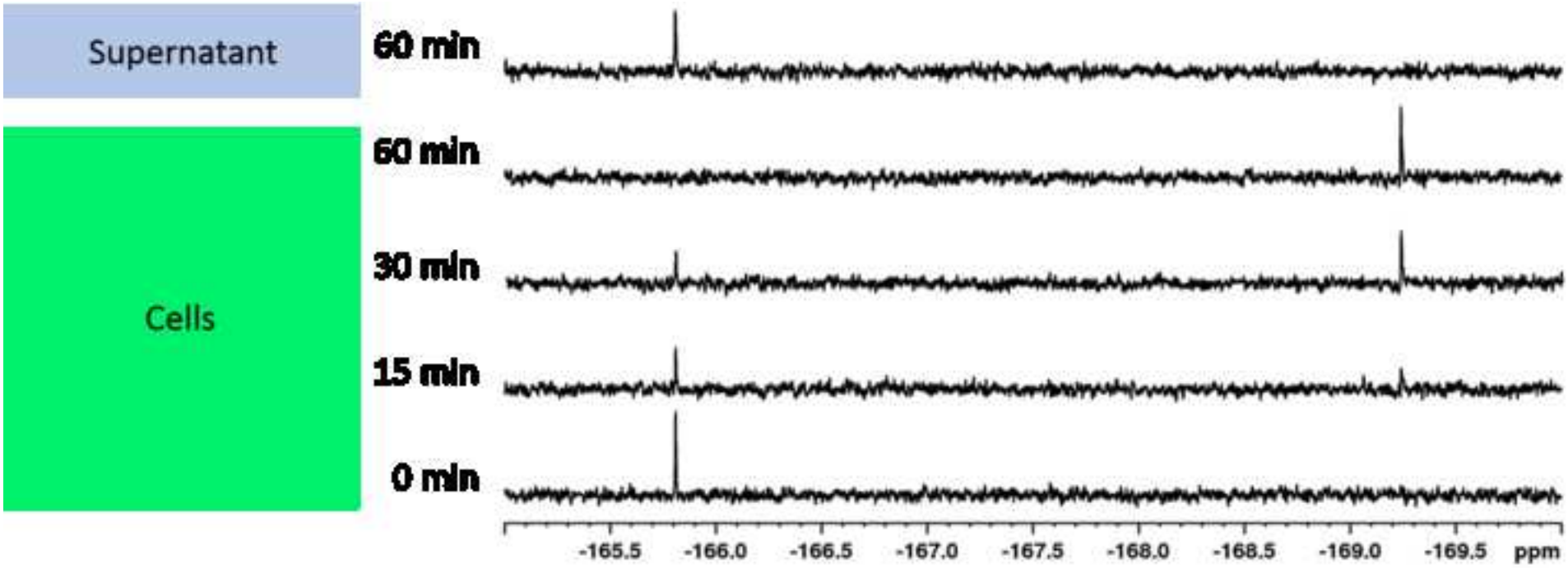

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Name of Reagent/Equipment	Company	Catalog Number	Comments/Description
AGNH	Purified in-house	N/A	TVAG_213720
UNH	Purified in-house	N/A	TVAG_092730
Adenosine	Sigma	A9251	
5-Fluorouridine	Sigma	F5130	
Dimethyl sulfoxide-D6	Cambridge Isotope Labs	DLM-10-100	D, 99.9%
Potassium phosphate monobasic	Sigma	P0662	
Potassium phosphate dibasic	Sigma	P3786	
Potassium chloride	Sigma	P9541	
Deuterium oxide	Cambridge Isotope Labs	DLM-4-100	D, 99.9%
Hydrochloric acid	Fisher Chemical	A144212	
Triton X-100	Sigma	X100	
3-(Trimethylsilyl)propionic-2,2,3,3,-d4 acid sodium salt (TSP)	Sigma	269913	D, 98%
2,2,2-Trifluoroethanol-1,1-d2	Sigma	612197	D, 99.5%
Pipette	Gilson	F123602	PIPETMAN Classic P1000
Pipette	Gilson	F123601	PIPETMAN Classic P200
Pipette	Gilson	F123600	PIPETMAN Classic P20
Microfuge tubes	Fisher Scientific	05-408-129	
Conical tubes	Corning	352099	
Microcentrifuge	Eppendorf	5418	
Vortex mixer	Fisher Scientific	02215365	
NMR tubes	Norell	502-7	Or as appropriate for the NMR
NMR spectrometer	Bruker	N/A	AvanceIII500
Prism software	GraphPad	N/A	Version 5.04

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April 18, 2019

Bing Wu, Ph.D.
Review Editor
Journal of Visualized Experiments

Dear Dr. Wu:

The 3rd revised manuscript titled 'NMR-based activity assays for determining compound inhibition, IC_{50} values, artifactual activity, and whole cell activity of nucleoside ribohydrolases' authored by Brian J. Stockman, Abinash Kaur, Julia K. Persaud, Maham Mahmood, Samantha F. Thuilot, Melissa B. Emilcar, Madison Canestrari, Juliana A. Gonzalez, Shannon Auletta, Vital Sapojnikov, Wagma Caravan, and Samantha N. Muellers is being submitted for publication in *JoVE Chemistry*. The following changes have been made in response to editorial comments:

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3. I have combined a number of shorter steps while still trying to maintain clarity.

4. I reduced the amount of text in the longer steps as much as possible.

Sincerely,

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