Application Note

Characterizing a Better Uncoupler

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Key Features

- The novel compound BAM15 uncouples oxidative phosphorylation, depleting mitochondrial membrane potential at a wider effective concentration range than the classical uncoupler FCCP.
- BAM15 and FCCP uncoupling properties are compared in HCT116, C2C12, and Huh7 cell lines using the Seahorse XF assay to determine maximal oxygen consumption rate values in the presence of an ATP synthase inhibitor.
- With proper uncoupler titrations, BAM15 offers more flexibility compared to FCCP when performing optimization experiments with valuable samples.

Introduction

An uncoupler, when used in the context of mitochondrial biology, is any substance that "uncouples" oxidative phosphorylation, or more specifically, the electron transport chain (ETC) from ATP synthase. Uncoupling is accomplished through the dissipation of the mitochondrial membrane potential ($\Delta\psi_M$) without the generation of ATP, resulting in either full or partial mitochondrial depolarization. The loss of membrane potential resulting from uncoupling leads to the generation of heat. Uncouplers exist as proteins (UCPs) found in brown adipose tissue, and as pharmacologic agents such as the classical uncouplers carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and 2,4-dinitrophenol (DNP).

The experimental use of classical uncouplers has played an integral role in determining mitochondrial function, yet they can be difficult to use due to variability between systems and cell lines. For example, FCCP has been increasingly used in cell systems as a means of determining maximal oxygen consumption rate (OCR). This works well, provided that the FCCP concentration has been optimized for the specific cell line, as the effective dose range of FCCP is typically very narrow. If the optimal concentration of FCCP has not been determined, one runs the risk of not adding enough FCCP, resulting in a submaximal OCR, or adding too much FCCP, which can reduce OCR.

The mechanism by which FCCP inhibits oxygen consumption has been attributed to plasma membrane depolarization, shift in the oxygen sensitivity of the ETC, and hydrolysis of cellular ATP resulting from oxygen depletion.[1-3] Effectively titrating the FCCP concentration can mitigate this negative effect. However, this can prove difficult when working with small amounts of starting material, such as primary cell lines. To provide a wider effective dose range for determining maximal OCR, the novel uncoupler BAM15 was developed and described in a recent publication.[4] In this application note, the uncoupling properties of BAM15 are measured in parallel with the classical uncoupler FCCP in three different cell lines. Our results reinforce those previously published, showing that BAM15 is more amenable to determining maximal OCR values and has a broader effective concentration.

Methods

Cell Culture

All cell lines (HCT116, C2C12, Huh7) were cultured in accordance with standard practice apart from HCT116 cells which were optimized for growth in DMEM. C2C12 cells were passaged at 75% confluency in order to maintain the myoblast properties of the cell line.

XF Assay

All cells were plated at a density of 10,000 cells/well (optimal density determined empirically) and cultured for 12 hours in DMEM at 37°C, 5% CO_2. On the day of the experiment, cell culture DMEM was replaced with Seahorse XF DMEM, pH 7.4 (Agilent Part No. 103575-100) supplemented with 10 mM glucose (Seahorse XF 1.0 M Glucose Solution, Agilent Part No. 103577-100), 1 mM pyruvate (Seahorse XF 100 mM Pyruvate Solution, Agilent Part No. 103578-100), 2 mM glutamine (Seahorse XF 200 mM Glutamine Solution, Agilent Part No. 103579-100). Cells were imaged/degassed using the Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek Instruments). All chemicals unless otherwise indicated were from Cayman Chemical: BAM15 (Cayman Item No. 17811), oligomycin complex (Cayman Item No. 11341), FCCP (Cayman Item No. 15218), TMRE, and Hoechst stain. Final concentrations for all reagents used in the XF experiments were as follows: oligomycin, 1 µg/ml; FCCP and BAM 15, as indicated; Hoechst stain, 1.6 µM; and TMRE, 20 nM.

Imaging

At the completion of the XF experiment, cell plates were removed from the XF analyzer and placed in the Cytation™ 5 Cell Imaging Multi-Mode Reader for normalization using Agilent's Cell Normalization Software. Following normalization, plates were washed 3x with Hank's Balance Salt Solution and imaged using the Cytation™ 5 (523 LED/RFP filter cube). TMRE fluorescence was quantified using Gen5 Imaging Software (BioTek Instruments).

Results

When titrating FCCP and BAM15 in different cell lines, it immediately became apparent that different cell lines responded differently to the two reagents (**Figure 1**). To test the degree of uncoupling, experiments were set up in two different ways. The first, a classical "stress test," injected uncoupler following an initial oligomycin injection. Oligomycin, which is an inhibitor of the ATP synthase, decreases OCR to the basal, non-phosphorylating rate. With the injection of uncoupler, OCR increases due to the dissipation of $\Delta\psi_M$. When $\Delta\psi_M$ is fully dissipated, OCR should theoretically plateau. In the second setup, the uncoupler is added first, followed by oligomycin. Here, the uncoupler increases OCR by dissipating $\Delta\psi_M$, thus uncoupling oxidative phosphorylation. If fully uncoupled, OCR should not respond to oligomycin. Any oligomycin response would suggest that either the oligomycin is causing cellular toxicity, or that further optimization is required. In both experimental setups, TMRE and Hoechst stain were added for measurement of membrane potential and cell number, respectively. The results from these experiments in HCT116, C2C12, and Huh7 cells are shown below.

The response of OCR in HCT116 cells to different concentrations of uncoupler is shown in **Figure 1**. BAM15 is shown on the top and FCCP on the bottom. In this cell line, BAM15 was well tolerated at concentrations up to 8 µM, compared with FCCP, which began to inhibit OCR at concentrations between 1-2 µM. These results show that BAM15 is relatively well tolerated in this cell line, whereas FCCP requires careful titration to prevent inhibition of OCR.

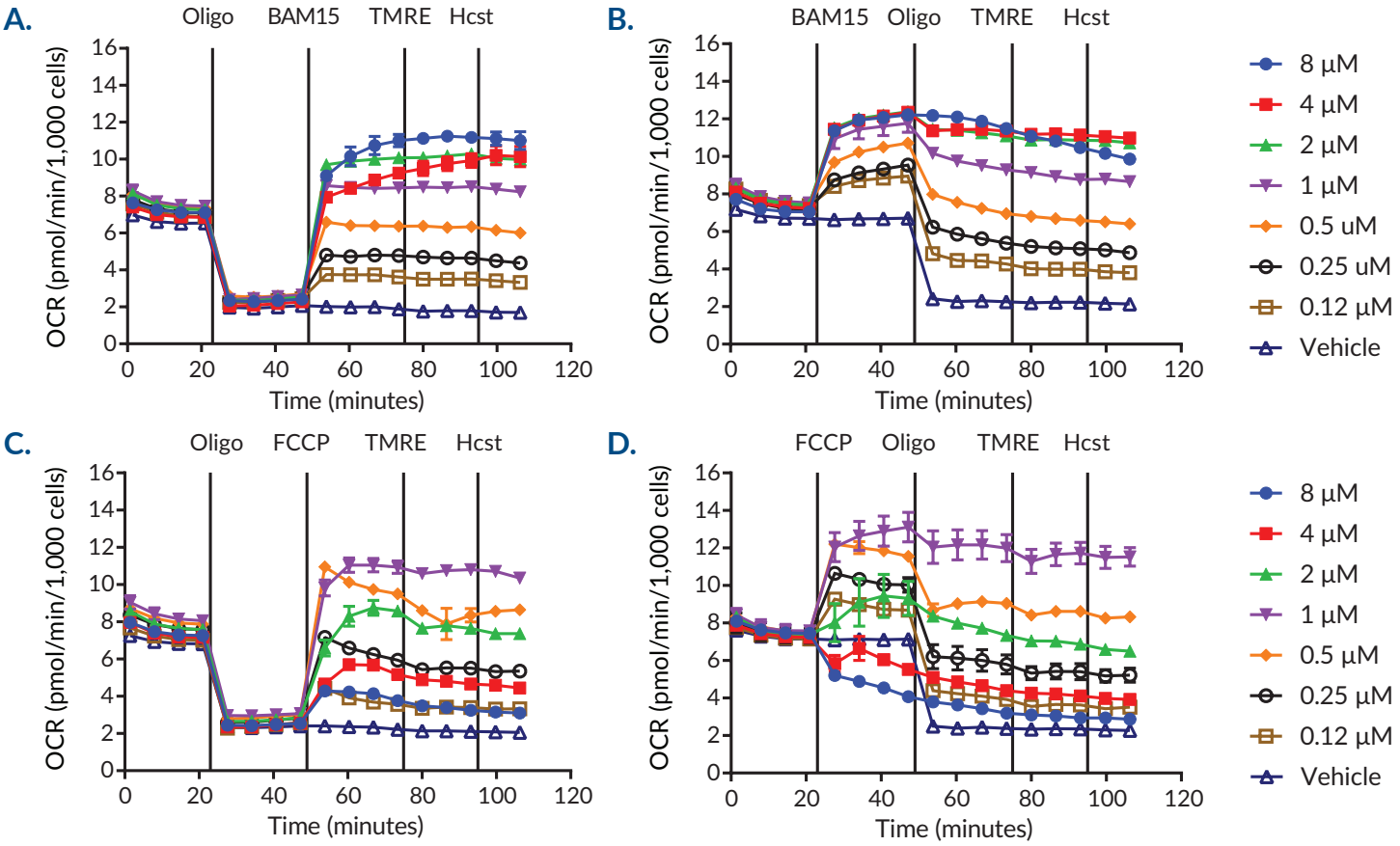

Figure 1. A. HCT116 cells treated with oligomycin followed by BAM15 show maximal OCR values at 8 µM BAM15. This concentration is sufficient to maximize OCR with minimal toxicity. **B.** Treatment with BAM15 followed by oligomycin results in a minimal response to OCR upon the addition of oligomycin. **C.** Cells that are treated with oligomycin followed by FCCP show maximal OCR values at 1 µM FCCP. Higher concentrations of FCCP result in decreases in OCR. **D.** Treatment with FCCP followed by oligomycin shows a slight response to oligomycin at 1 µM. Concentrations >1 µM result in decreases in OCR.

The titration of BAM15 and FCCP in C2C12 cells is shown in **Figure 2**. In this experiment, BAM15 increased OCR values to concentrations up to 2.5 µM, above which toxicity is observed. At 2.5 µM, no inhibition is observed following the addition of oligomycin, indicating that the mitochondria are fully uncoupled. Inhibition of OCR by oligomycin at concentrations greater than 2.5 µM BAM15 is likely due to cellular toxicity as indicated by the gradual decline in OCR following each addition.

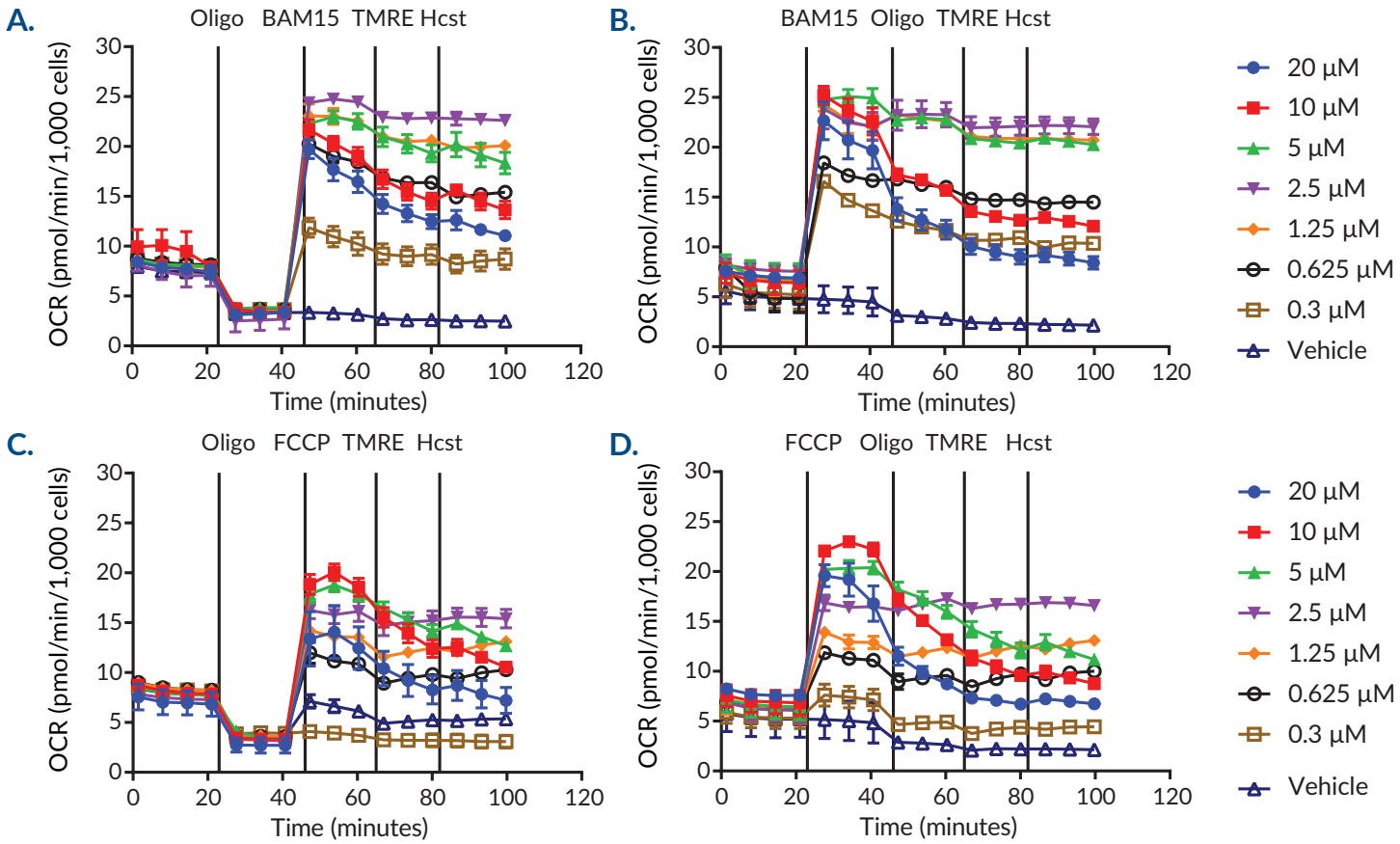

Figure 2. A. C2C12 cells treated with oligomycin followed by BAM15 show maximal OCR values at 2.5 µM BAM15. This concentration is sufficient to maximize OCR with minimal toxicity. Concentrations >5 µM resulted in gradual decreases in OCR suggesting toxicity. **B.** Treatment with BAM15 followed by oligomycin results in a minimal response to OCR upon the addition of oligomycin at 2.5-5 µM BAM15. **C.** Cells treated with oligomycin followed by FCCP show maximal OCR values at 10 µM FCCP, followed by a gradual decrease in OCR indicating toxicity. Higher concentrations of FCCP result in decreases in OCR. **D.** Treatment with FCCP followed by oligomycin shows a slight response to oligomycin 0.625 µM with minimal response observed from 1.25-2.5 µM. Concentrations >2.5 µM result in decreases in OCR.

In the case of FCCP, a similar optimal concentration of 2.5 µM is required for increasing OCR without toxicity, yet the maximal OCR observed at this concentration is not as high as that observed with BAM15. This observation suggests that the optimal concentration of FCCP required for C2C12 cells may lie within the range of 1.25 to 2.5 µM. Further titration would therefore be required.

Figure 3 illustrates the OCR response in Huh7 cells to the uncouplers. This cell line has demonstrated the highest degree of sensitivity to FCCP as illustrated in the lower half of the figure. In previous experiments, the optimal concentration of FCCP was determined to be 500 nM (data not shown). In this set of experiments, FCCP begins to inhibit OCR and show signs of cellular toxicity around 2.5 µM. When titrating BAM15, the optimal concentration range was found to be 1.25 to 2.5 µM. Inhibition of OCR does not start to occur until BAM15 concentrations exceed 2.5 µM.

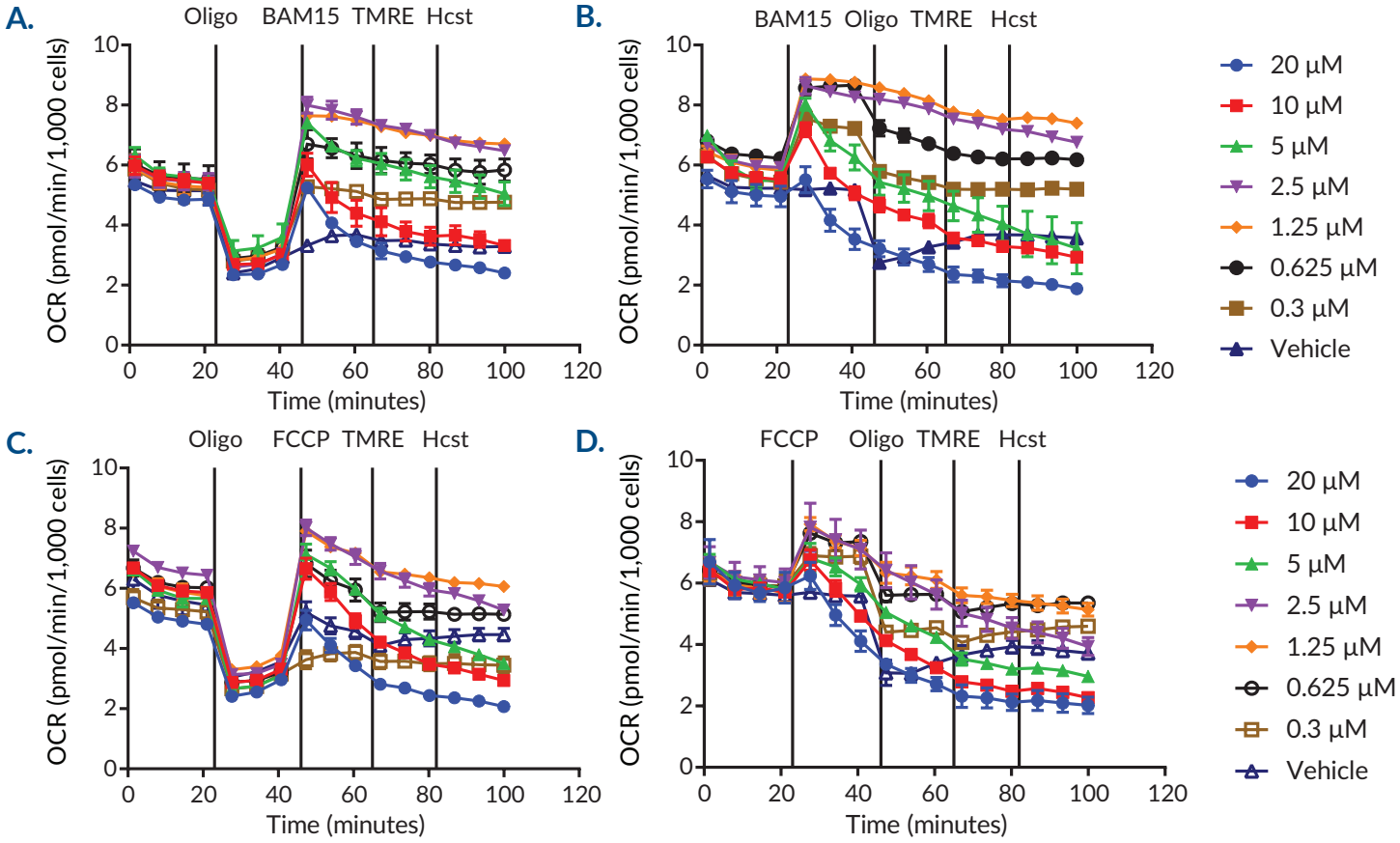

Figure 3. A. Huh7 cells treated with oligomycin followed by BAM15 show maximal OCR values at 1.25-2.5 µM BAM15. This concentration is sufficient to maximize OCR with minimal toxicity. Concentrations >2.5 µM resulted in gradual decreases in OCR suggesting toxicity. **B.** Treatment with BAM15 followed by oligomycin results in a minimal response to OCR upon the addition of oligomycin at 2.5-5 µM BAM15. **C.** Cells treated with oligomycin followed by FCCP show maximal OCR values at 10 µM FCCP, followed by a gradual decrease in OCR indicating toxicity. Higher concentrations of FCCP result in decreases in OCR. **D.** Treatment with FCCP followed by oligomycin shows a slight response to oligomycin 0.625 µM with minimal response observed from 1.25-2.5 µM. Concentrations >2.5 µM result in decreases in OCR.

Concentration response curves measuring the third timepoint after injection of uncoupler (in the "stress test" experiments) can be found in **Figure 4**. These curves best illustrate the performance profiles of the uncouplers in the three different cell lines. It is worth noting that the differences among the vehicle-treated controls for each graph corresponds to the varied basal OCR for each cell type. The difference between the maximal OCR and baseline would be the "spare capacity" of each cell line.

To determine that membrane potential was depleted at the concentrations tested, cells were stained with TMRE and imaged. An example image is shown in **Figure 5** along with concentration response curves of TMRE fluorescence *versus* uncoupler concentration. These curves show that the sum of TMRE positive objects/cell approaches zero at concentrations ~1 µM with both uncouplers.

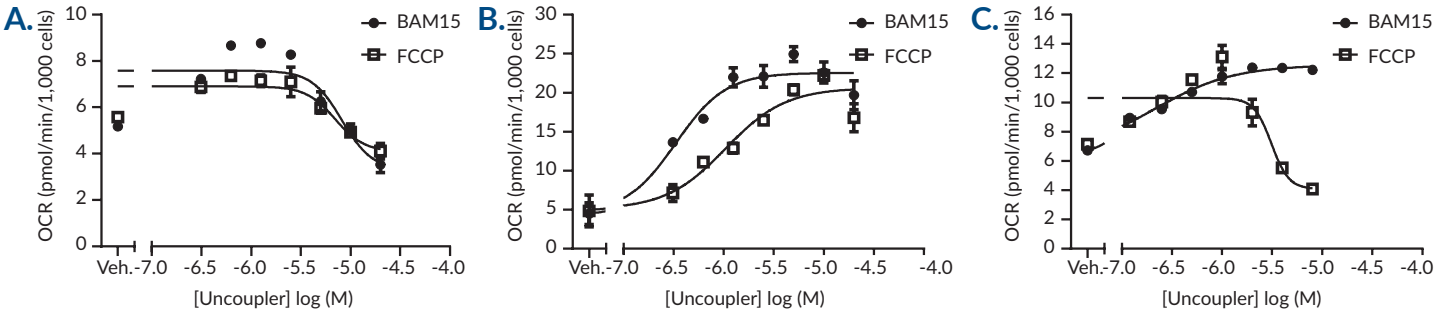

Figure 4. The response of **A.** HCT116, **B.** C2C12, and **C.** Huh7 cells to different concentrations of uncoupler. Data was sampled at measurement point 10 from plots A and C from Figures 1-3.

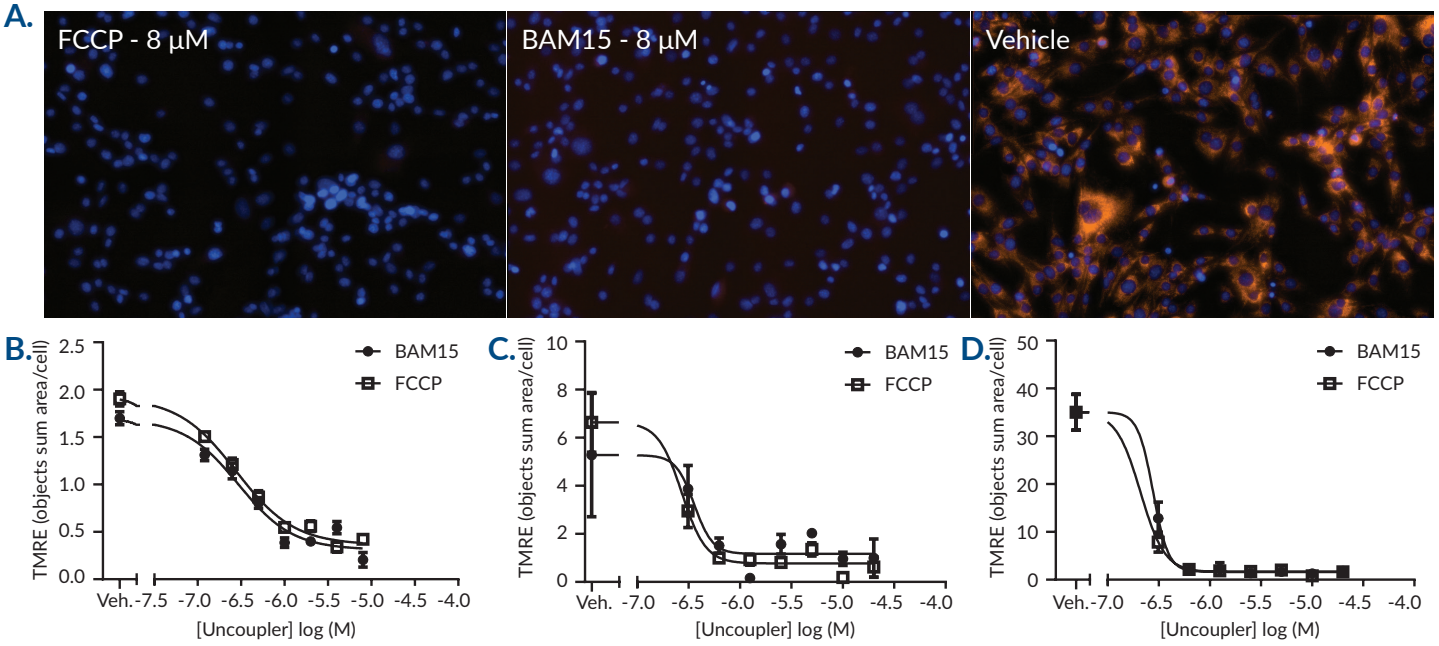

Figure 5. XF plates were imaged following the completion of each assay in order to measure TMRE fluorescence. **A.** Example images are shown above. **B-D.** Concentration response curves of TMRE fluorescence *versus* uncoupler for HCT116, C2C12, and Huh7 cells, respectively.

Conclusion

Taken together, these data show that BAM15, when compared to FCCP, facilitates maximal OCR at a wider range of concentrations. Additionally, cells appear to be more tolerant to BAM15 *versus* FCCP, as indicated by the consistently high OCR values following injection of additional downstream compounds. While BAM15 does not fully negate the need for proper uncoupler titrations, it does offer more flexibility when performing optimization experiments with valuable material.

References

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4. Kenwood, B.M., Weaver, J.L., Bajwa, A., *et al.* Identification of a novel mitochondrial uncoupler that does not depolarize the plasma membrane. *Mol. Metab.* **3(2)**, 114-123 (2013).

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