Application Note

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Cell-Based Screening for Drug-Induced Mitochondrial Toxicity

Using the Agilent MitoXpress Xtra oxygen consumption assay

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Abstract

Drug-induced mitochondrial dysfunction is an important contributor to compound attrition and postmarket drug withdrawals across various drug classes. The importance of the mitochondrial network as a site for off-target drug effects highlights the need for *in vitro* assays capable of detecting drug-induced mitochondrial liabilities. Conventional cytotoxicity assays are, however, particularly unsuited to this task due to the ability of relevant cell lines to circumvent the down-stream implications of perturbed mitochondrial function. This Application Note describes how the Agilent MitoXpress Xtra oxygen consumption assay can be used to address this deficit. The assay is microplate-based, and measured on compatible fluorescence plate readers, delivering a direct, cell-based solution for the convenient identification of drug-induced mitochondrial dysfunction and associated dose-response analyses. The potential for multiplexed assessments of cytotoxicity and mitochondrial function to provide further contextualization of a drug-induced mitochondrial insult is also outlined.

Introduction

Mitochondria are key regulators of cellular function and energy supply, such that functional perturbation can have deleterious consequences for the cell. There has been a growing awareness of the importance of the mitochondrial network as a site for the off-target effects of drug therapy. This focus has been driven, in large part, by the implication of mitochondrial dysfunction in drug-induced organ toxicities, late-stage attrition, black-box warnings, and market withdrawals.[1,2] These implications have highlighted the critical importance of deploying effective *in vitro* strategies for the identification of such mitochondrial liabilities early in the discovery process.[4] A primary challenge, however, is that the combination of conventional cytotoxicity assays and typically deployed cell lines are wholly unsuited to this task, due to the ability of these cell lines to mitigate the consequences of mitochondrial insult.[3,4] This issue can be addressed through more direct interrogation of mitochondrial function, with *in vitro* measurement of mitochondrial oxygen consumption, in whole cells or isolated mitochondria, widely considered the gold-standard approach.[4,5] Traditionally, such analyses have been performed using conventional polarography, however, low throughput coupled with the necessity for specialized equipment and expertise limits its practicality when applied to large compound libraries.

The Agilent MitoXpress oxygen consumption assay addresses these constraints by harnessing oxygen-sensitive fluorescent probes, which facilitate convenient, direct interrogation of mitochondrial function in microplate format. This assay combines the convenience of conventional cytotoxicity assays with the specificity of polarographic oxygen consumption measurements. This Application Note describes how the assay can be used to screen for drug-induced mitochondrial dysfunction using HepG2 cells, and how supplementary multiplexing can provide further context to observed metabolic perturbation.

Agilent MitoXpress Xtra oxygen consumption assay

The MitoXpress Xtra oxygen consumption assay facilitates convenient, fluorescence-based, microplate compatible interrogation of mitochondrial function by monitoring the oxygen depletion due to aerobic metabolism. The kit contains a water-soluble, fluorescent oxygen probe that shows reduced fluorescence signal in the presence of oxygen. Cells are grown in standard 96-well plates, and are overlaid with media containing the MitoXpress Xtra probe. Each well is then covered with a sealing layer of HS oil to limit oxygen back-diffusion. As cellular respiration reduces the concentration of dissolved oxygen in each well, probe signal increases (Figure 1A). This change in signal is measured kinetically on a fluorescence plate reader, and is indicative of the rate of cellular respiration. When cells are treated with modulators of mitochondrial respiration, the rate of signal change will either decrease (if mitochondrial respiration is inhibited) or increase (if respiration is increased or the electron transport chain is uncoupled from ATP production) as outlined in Figure 1B.

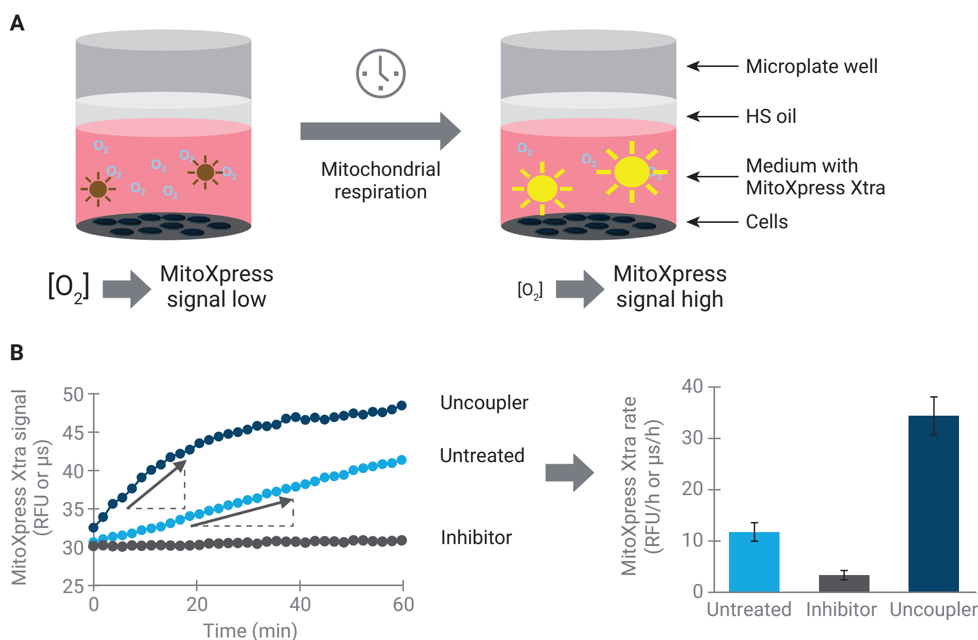

Figure 1. Measuring mitochondrial function using the Agilent MitoXpress Xtra oxygen consumption assay. (A) MitoXpress Xtra is added to cells and overlaid with HS oil. Cellular respiration leads to oxygen depletion in the sample, resulting in increased MitoXpress Xtra signal. (B) This change in signal is measured kinetically on a fluorescence plate reader. The rate of signal increase is calculated by linear regression (slope calculation), and is indicative of the rate of cellular respiration.

Assay workflow overview

The MitoXpress Xtra oxygen consumption assay uses a simple 'mix-and-measure' workflow to assess mitochondrial respiration in live cells. Cells grown in standard 96-well plates are cultured and treated with drugs as required. On the day of measurement, the culture medium is exchanged to fresh culture medium or the assay medium of choice containing the MitoXpress Xtra probe at the recommended concentration. The assay is compatible with a range of commonly used media for mammalian cell culture (DMEM, MEM, RPMI, primary hepatocyte medium, and others), and can be used in the presence of fetal bovine serum (FBS) or phenol red. To reduce oxygen diffusion from the environment into the well, the wells are overlaid with a layer of HS oil, and the plate is immediately transferred into a plate reader for kinetic measurement of the MitoXpress Xtra signal over 90 to 120 minutes, depending on the cell type used (Figure 2). The resulting kinetic data are then analyzed using pre-optimized, onboard analysis templates developed for selected plate-readers or using the Agilent MitoXpress Xtra Data Visualization Tool. See the References and Additional Resources sections for extra links and information.

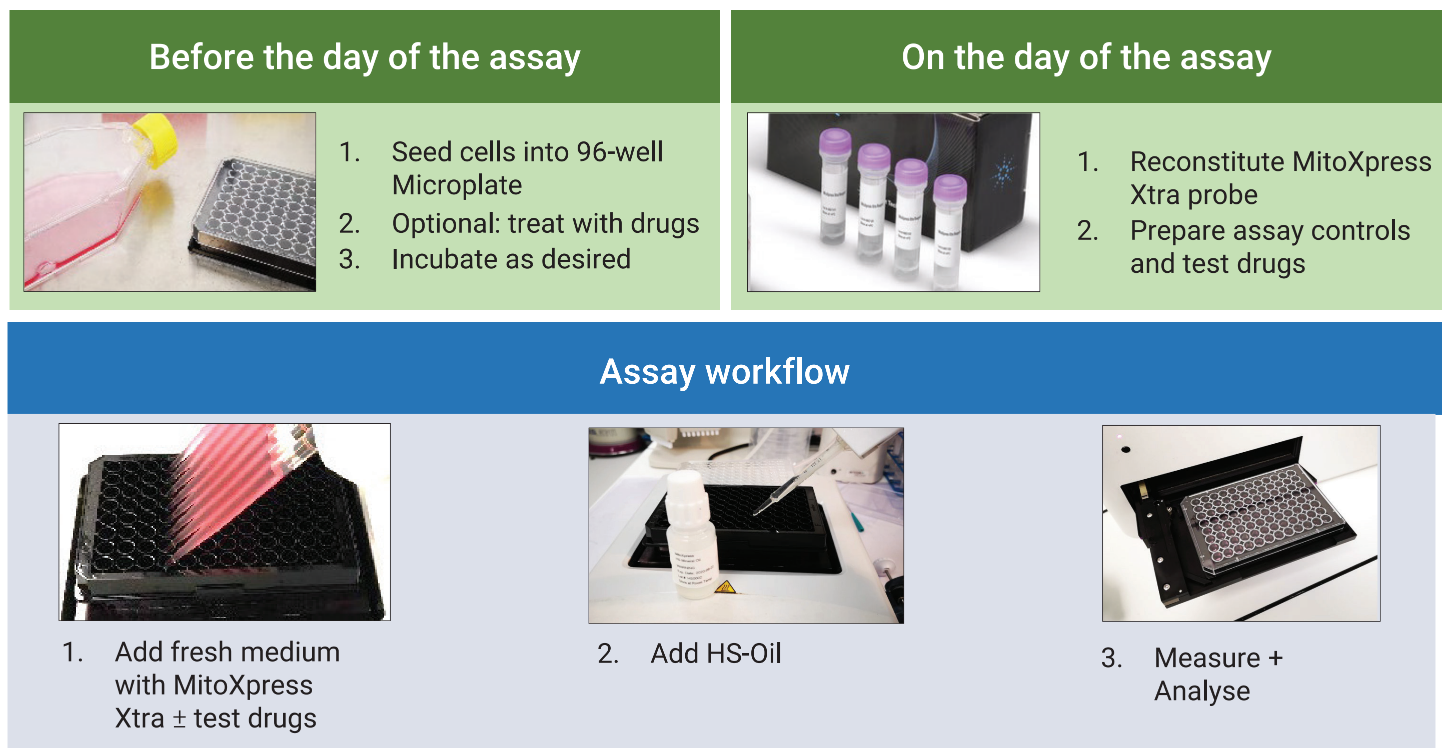

Figure 2. The workflow of the Agilent MitoXpress Xtra oxygen consumption assay using an adherent cell line.

Material and Methods

Cell culture and plating

HepG2 cells were maintained in DMEM (Gibco, #41965039) supplemented with 10% FBS (Sigma, #F0804) and 1% Pen/Strep solution (Sigma, #P4333). Before the assay, cells were seeded at 30,000–80,000 cells/well (dependent on treatment duration) into TC-treated 96-well plates (Sarstedt, #655090CI) and allowed to adhere overnight (5% CO_2, 37 °C) before treatment with drugs or vehicle at the indicated time points.

Plate preparation

Note: All media, reagents, and the plate-reader should be prewarmed to 37 °C.

- Agilent MitoXpress Xtra (part number MX-200-4) reagent was reconstituted in 1 mL growth medium then diluted in 10 mL prewarmed growth medium as described in the MitoXpress Xtra user guide.[8]
- For measurements multiplexed with Calcein AM, 1.25 µm of the dye was added to the medium.
- The plate was removed from the incubator and placed on a plate-block heater set to 37 °C. Cell growth medium was replaced in each well with 100 µL of the MitoXpress Xtra reagent in medium.
- Where applicable, 1 µL of drug stock (100× in DMSO) or vehicle was added to each well.
- Wells were then sealed by overlaying with 100 µL prewarmed HS oil (included in the kit) to limit oxygen back-diffusion into the sample using a repeater pipette.

Instrument settings, data acquisition, and reduction

- The plates were then immediately measured kinetically on a CLARIOstar (BMG Labtech) plate reader (prewarmed to 37 °C) with a TRF-Optical attachment. Dual-read time-resolved fluorescence was measured for 90 to 120 minutes using minimal interval time between individual reads. The probe was excited at 340 ±50 nm (TREX-L filter), and emission was measured at 655 ±25 nm (655-BP25 filter) using delay/window times of 30 µs/0 µs, and 100 µs/30 µs. For measurements multiplexed with Calcein AM, fluorescence intensity was measured using the following settings: Ex: 483 ±14 nm, Ex: 530 ±30 nm, and dichroic: 502.5 after 30 minutes
- Calculation of fluorescence lifetime and data analysis was performed as described in the MitoXpress Xtra user guide,[8] using MARS software (BMG Labtech) data analysis templates. The time-resolved intensity values for both measurement windows were blank corrected and lifetime was calculated. Kinetic data analysis was performed by performing linear regression over the linear part of the kinetic data.
- For this and selected other plate-readers, predefined instrument protocols with optimum measurement settings and predefined data analysis templates can be downloaded from the Agilent Cell Analysis home page or requested from cellanalysis.support@agilent.com. Additional information on recommended settings and data reduction can be found in the MitoXpress Xtra oxygen consumption assay user guide.[8]
- *Comparable plate-readers from other vendors, for example Biotek's Cytation 1/5 or Synergy H1/Neo2 can also be used with the Agilent MitoXpress Xtra Assay.* Visit https://www.agilent.com/en/products/cell-analysis/plate-reader-compatible-assays for further information on suitable plate-readers.

The MitoXpress Xtra Data Visualization Tool is also available for download to aid data interpretation.

Results and discussion

Assay performance

To assess assay consistency, basal MitoXpress Xtra rates of HepG2 cells were compared to rates derived from Antimycin A treated cells (1 µm). The average rates and standard deviations from these comparative rates were used to calculate Z' factors as a measure of response consistency.[6]

Figure 3A shows representative MitoXpress Xtra signal profiles for a standard 96-well plate. After linear regression, the MitoXpress Xtra rates (µs/h) were calculated for both basal and Antimycin A treated cells. A robust signal to ratio and uniformity among wells were observed, translating to a Z' factor of >0.6 (depending on cell line/density and cell seeding consistency).

Screening for mitochondrial toxicants in HepG2 cells

The MitoXpress Xtra assay was used to identify compounds with mitochondrial liabilities using a workflow to screen for acute effects on mitochondrial function. Several compounds with known effects on mitochondrial respiration were screened at single concentrations using HepG2 cells. Drugs were added to the wells immediately before the sample was overlaid with HS oil.

Figure 4 shows a subset of the results obtained. Nimesulide and FCCP were correctly identified compounds leading to increased respiration, while the other drugs tested reduced mitochondrial respiration, as previously reported in the literature.[3,7]

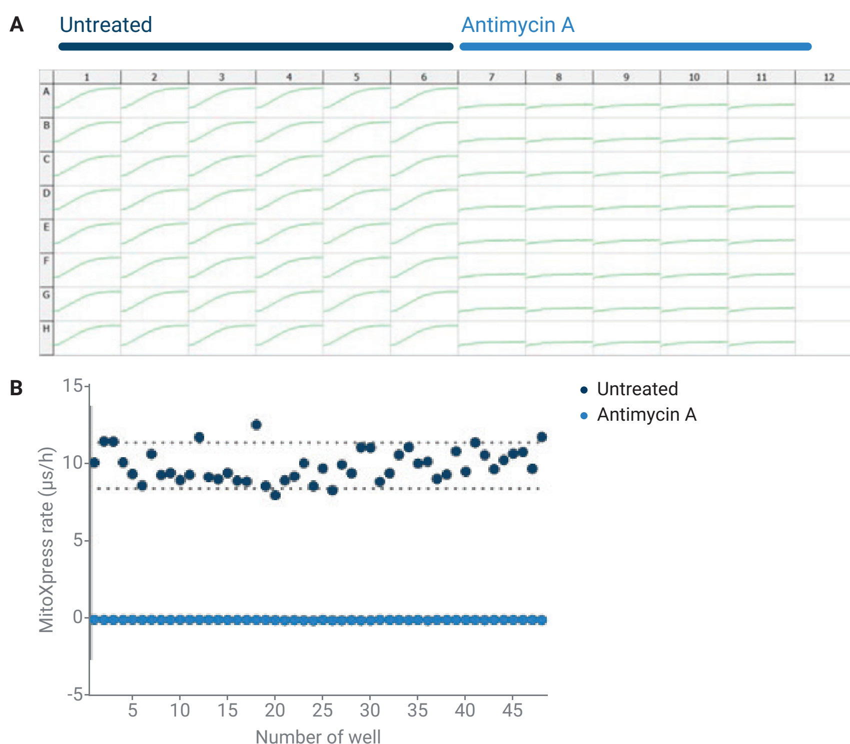

Figure 3. Assay performance of the Agilent MitoXpress Xtra assay in HepG2 cells. (A) Kinetic MitoXpress Xtra data of untreated and Antimycin A (1 µm) treated cells B) Sample result from Z' factor analysis. MitoXpress Xtra rates of Antimycin A treated HepG2 cells were compared to rates from untreated cells, revealing a Z' factor of 0.67. Dotted lines represent ±15% of the average MitoXpress Xtra rate.

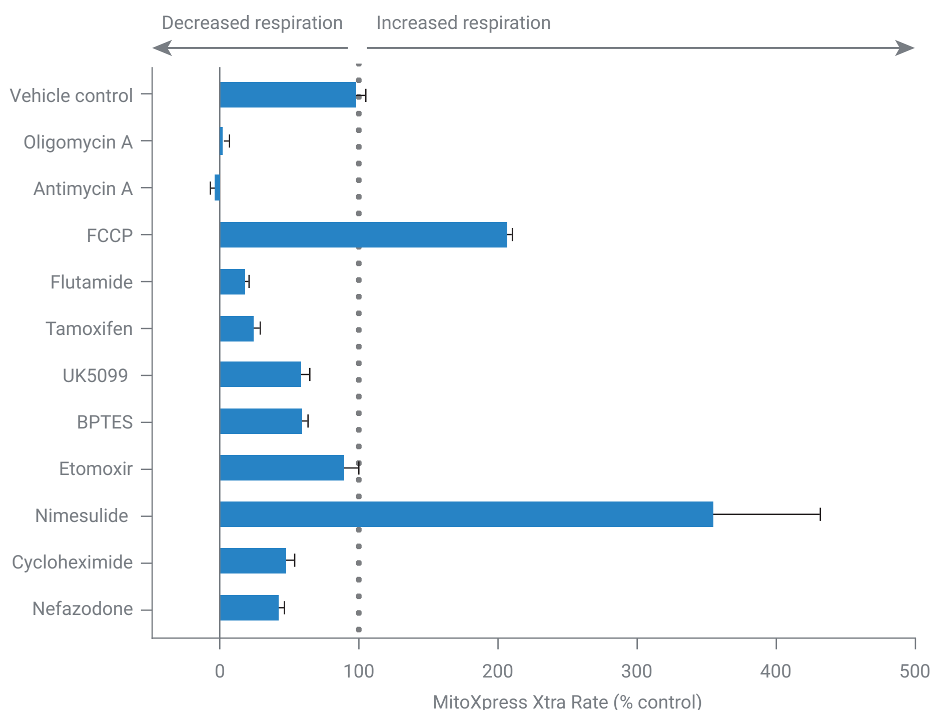

Figure 4. Agilent MitoXpress Xtra rates in HepG2 cells treated acutely with single concentrations of drugs (Oligomycin A: 1.67 µm; Antimycin A: 0.67 µm; FCCP: 1.67 µm; Flutamide: 16.7 µm; Tamoxifen: 16.7 µm, UK5099: 4 µm; Etomoxir: 8 µm; Nimesulide: 16.7 µm; Cycloheximide: 16.7 µm; Nefazodone: 16.7 µm). Data were normalized to rates from vehicle-treated cells. MitoXpress Xtra rates >100% indicate an increase in respiration; rates <100% indicate inhibition of respiration.

These results demonstrate the utility of the MitoXpress Xtra assay for compound screening designed to detect the acute impact of drug treatment on cells. The simple workflow allows for the rapid screening of large numbers of drugs, and can differentiate drugs with an inhibitory effect on mitochondrial respiration from those that increase mitochondrial respiration.

Dose-response analysis

Compounds that elicit changes in the single-concentration screening described previously can be further characterized by establishing dose-response relationships, thereby enabling more accurate compound ranking. As an example, HepG2 cells were treated with serial dilutions of Flutamide, Nefazodone, and Nimesulide immediately before measurement. These compounds have been reported as exhibiting mitochondrial liabilities, received black-box warnings,[2] and showed a response in the single concentration-screening described earlier. Flutamide and Nefazodone exhibited a clear dose-dependent reduction in mitochondrial respiration, while the Nimesulide caused a dose-dependent increase of respiration (Figure 5). These results are in agreement with their reported activity as electron transport chain inhibitors or uncoupler, respectively. These data facilitate the convenient calculation of IC_{50}/EC_{50} values, enabling compound ranking and delivering a more comprehensive determination of the impact of drug treatment on mitochondrial function.

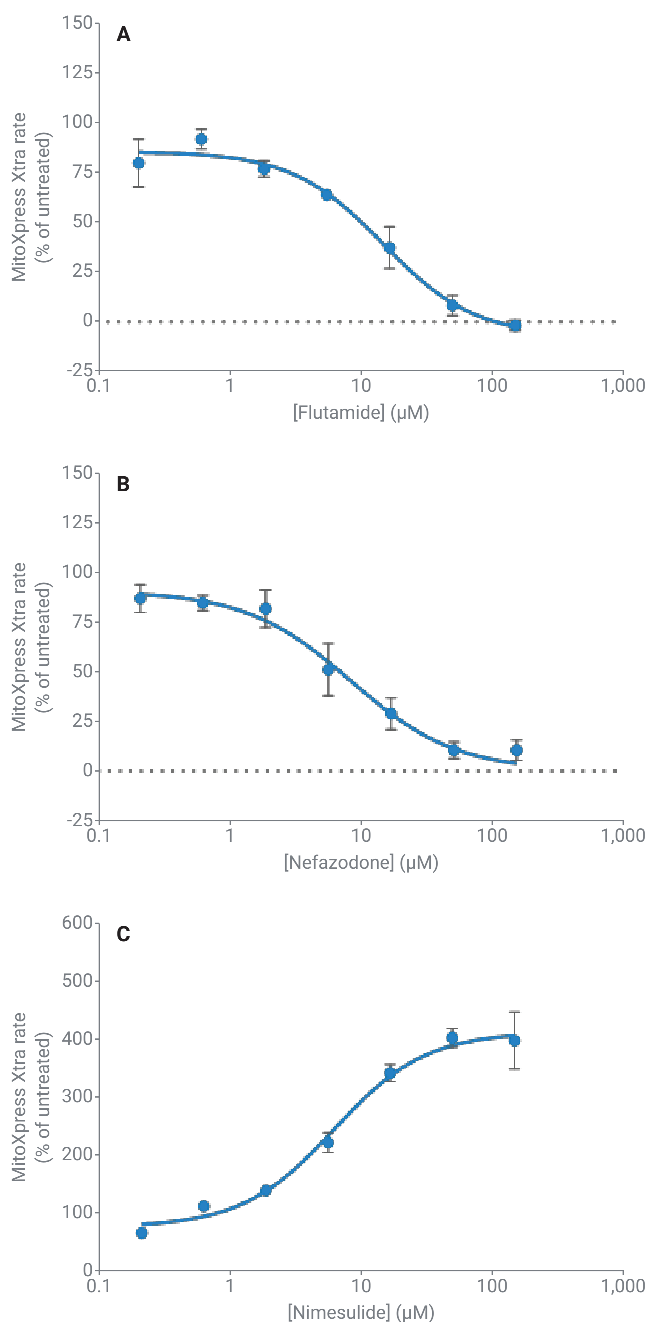

Figure 5. Dose-response data generated using the Agilent MitoXpress Xtra assay after 24 h treatment with increasing concentrations of model drugs. Flutamide and Nefazodone reduced mitochondrial respiration, while the uncoupler Nimesulide led to an increase in respiration. Data were normalized to rates from vehicle-treated cells.

Multiplexed assessment of mitochondrial function and cytotoxicity

Longer-term treatments are performed to reveal drug-toxicity mediated by bioactivation, bioaccumulation, or by impairment of mitochondrial biogenesis or turn-over. For this, the workflow described previously was applied to cells pretreated with the test compounds for 24 hours before measurement. However, after extended exposure times, it can be difficult to determine whether any observed changes in cell respiration are a consequence of direct mitochondrial inhibition or nonspecific cytotoxicity. To better delineate drug-induced mitochondrial dysfunction and nonspecific toxicity after longer-term compound treatments, a multiplexed "mix-and-measure" approach including assays for both cytotoxicity and mitochondrial function should be applied.

Dose-response analyses of cell viability were measured by Calcein AM, and mitochondrial function was measured by MitoXpress-Xtra, for acute (0 hours) and longer-term (24 hours) treatments with two representative compounds, Flutamide and Tamoxifen (Figure 6). Upon acute treatment, both compounds induce a dose-dependent reduction in mitochondrial respiration, with no significant cytotoxicity observed, suggesting that both compounds have a direct inhibitory effect on mitochondrial function. After 24 hours treatment, however, Tamoxifen showed an additional decrease in cell viability, which was not observed for Flutamide, suggesting a multifactorial toxic response for Tamoxifen. These observations are consistent with the reported mode-of-actions.[4,5]

These differential dose responses highlight that common cytotoxicity assays are not well suited to the detection of mitochondrial liabilities. However, in combination with the measure of mitochondrial function using the MitoXpress Xtra assay, these orthogonal data sets can aid better contextualization of longer-term drug treatments, enabling more effective delineation of mitochondria-specific effects and nonspecific or multifactorial toxicity.

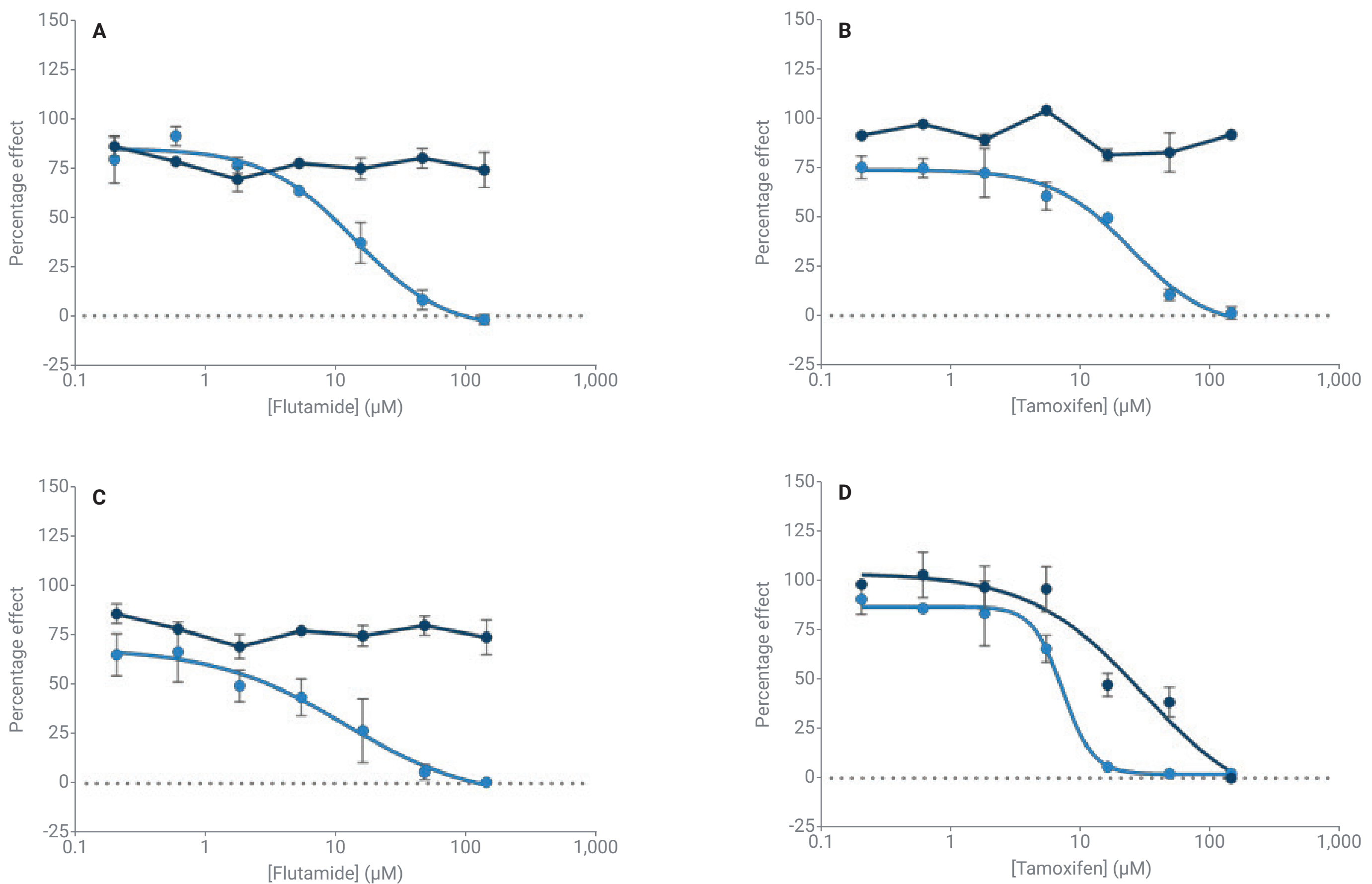

Figure 6. Multiplexed mitochondrial respiration (light blue) and viability (dark blue) assessment in HepG2 cells after treatment with increasing concentrations of Flutamide and Tamoxifen. Cells were treated acutely (upper plots) or for 24 hours (lower plots). Responses are shown as % of vehicle-treated cells.

Conclusion

Impaired mitochondrial function is a significant contributor to off-target drug effects, highlighting the need for effective *in vitro* strategies for the identification of metabolic liabilities early in the discovery process. These liabilities are not always reliably measurable using conventional cytotoxicity assays; however, *in vitro* oxygen consumption measurements using the Agilent MitoXpress Xtra assay provide a direct measure of mitochondrial function in live cells, facilitating the convenient detection of drug-induced mitochondrial perturbation. Cells are cultured in conventional 96-well plates and, using a mix-and-measure protocol, interrogated on a fluorescence plate reader to deliver the specificity and information content needed for such compound screening. This Application Note demonstrates that mitochondrial dysfunction can be identified with the requisite assay performance, and that drugs causing increases in respiration can be delineated from those that inhibit respiration in single concentration drug screens. This study also illustrates how dose-response relationships can be easily established to enable more effective compound ranking, and how multiplexed measurement of mitochondrial respiration and cytotoxicity are capable of better contextualizing the response of cells to longer-term compound treatments. The MitoXpress Xtra oxygen consumption assay is demonstrated to be a simple and valuable tool for the identification of drug-induced mitochondrial toxicity.

References

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8. MitoXpress Xtra Oxygen Consumption Assay user guide (https://www.agilent.com/cs/library/usermanuals/public/MitoXpress_Xtra_Oxygen_Consumption_Assay.pdf)

Additional resources

Agilent MitoXpress Xtra

https://www.agilent.com/en/products/cell-analysis/mitoxpress-ph-xtra-consumables/mitoxpress-xtra-oxygen-consumption-assay

The Agilent MitoXpress Xtra assay and pH-Xtra Data Visualization Tool is available from:

https://www.agilent.com/en/products/cell-analysis/mitoxpress-ph-xtra-consumables/mitoxpress-xtra-oxygen-consumption-assay#support

Instrument setup guide

https://www.agilent.com/en/products/cell-analysis/mitoxpress-ph-xtra-consumables/mitoxpress-xtra-oxygen-consumption-assay#literature

Downloadable instrument settings and data analysis templates for selected plate-readers are available from:

https://www.agilent.com/en/products/cell-analysis/plate-reader-compatible-assays

Further examples for how the MitoXpress Xtra oxygen consumption assay was used to screen for drug-induced mitochondrial dysfunction in cells and isolated mitochondria can be found at:

https://www.agilent.com/en/products/cell-analysis/mitoxpress-ph-xtra-consumables/mitoxpress-xtra-oxygen-consumption-assay#literature

www.agilent.com/chem

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