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Deliverable Report D8.2:

**Data set describing the impact of drug treatment on the mitochondrial function
of both 3D models**

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PUBLISHABLE SUMMARY

Data set describing the impact of drug treatment on the mitochondrial function of both 3D cardiac and liver cell models.

OBJECTIVES

To generate a Data set describing the impact of drug treatment on the mitochondrial function of both 3D models, using methods developed through Deliverable Report D8.1.

INTRODUCTION

The overall objective of this Task 8.2 is to generate data assessing the effect of chosen compounds on the mitochondrial function of chosen liver and cardiac cell models. The compounds, cell models are an output of WP5. Particular focus on mitochondrial function informing on cellular function, is presented in this deliverable, while an assessment of apoptosis induction and inflammatory markers is reported in specific deliverables. Methods were developed and adapted to facilitate such measurements and are described in detail in D8.1 report. In some instances these methods have been further developed during this reporting period and have been applied here to the assessment of drug treated liver and cardiac spheroid models.

Recent years have seen a growing appreciation for the importance of the mitochondrion as a site for off-target effects of drug therapy [1]. This is unsurprising when one considers both the multitude of sites where mitochondrial function can be perturbed and the deleterious consequences of such perturbation. Such drug-induced mitochondrial dysfunction has been shown to be a significant cause of drug-induced toxicity but detection of this toxicity in *in vitro* models has been hampered by the lack of suitable screening technologies. There has also been an increasing awareness of the limitations of standard tissue culture models due to their ability to circumvent mitochondrial insult, absence of critical metabolic pathways and an absence of cell-cell interactions contributing to a significant gap between *in vitro* model and the *in vivo* condition. The biological relevance of the *in vitro* observation therefore is heavily influenced by the model used. For liver studies, the gold standard for such measurements is primary human hepatocytes and additional biological relevance can be realised by culturing in 3D. This also offers the opportunity to co-culture primary human hepatocytes with non-parenchymal cells, including Kupffer cells, thereby better reflecting the cell composition of the human liver. Here we use scaffold-free 3-dimensional microtissues (spheroids) grown using the hanging drop method on GravityPLUS™ plates and transferred in GravityTRAP™ 96-well format for subsequent testing. The hepatocytes used are cryopreserved to address donor variability and access. The same measurement protocols will be used for testing spheroids generated from stem cell derived cardiomyocytes.

Oxygen is one of the most sensitive and direct indicators of mitochondrial dysfunction as it allows direct measurement of electron transport chain (ETC) activity. Using a water-soluble oxygen probe (MitoXpress®-Xtra), mitochondrial function can be assessed in a high throughput fashion on standard fluorescence plate readers. However when applied to spheroids, the small amount of biomaterial available presents a significant analytical challenge resulting in multiple spheroids being required per test well to generate measurable oxygen depletion. During the project we presented data (period 2 Annual Report) on the development and validation of a method capable of measuring single spheroids and the application of that method to the assessment of drug induced mitochondrial dysfunction of both hepatic and cardiac derived spheroids. The method devised and optimised facilitates single

spheroid measurements using low volume glass capillaries. This is a significant technical advance on the method described in MS7, and it was further continually applied to evaluate the compounds using the devised complex dosing scheme over the remaining time in the project to achieve the deliverable.

Human Liver Microtissues consisting of an organotypic co-culture of primary human hepatocytes and kupffer cells, and cardiac spheroids generated using iPS derived cardiomyocytes were delivered from InSphero *via* work package 5, as well as the relevant culture media, supplements, and environmental plate lids for long terms culture/incubations.

In this report data on 9 cardiac drug treatments (Idarubicin, Doxorubicin, Epirubicin, Daunorubicin, 5-fluorourcil, Amiodarone, MitoXanthrone, Docataxel, Paclitaxel) using available complex dosing schemes from work package 4 are presented for the cardiac model, while data on 4 liver drug treatments (Acetaminophen, Azathioprine, 5-fluorourcil, Phenytoin) using available dosing schemes of the hepatic spheroid model are also presented. At time of reporting, 1 other cardiac drug (Celecoxib) and a further 3 liver drugs (Isonazid, Valproic acid, and Cyclosporine) are being tested. As well as further 3 planned liver compounds being tested by both WP05 and WP08.

The analysis are carried out 7 days post initial treatment using the complex dosing scheme. This dosing scheme requires media exchange of the spheroid plates 3 times / day over 5 days, which requires media + drug preparation for 2 doses (therapeutic & Toxic) for 1 one or multiple drugs (if running in parallel) 3 times per day. This complex dosing regime is a resource intensive experimental design which only yields data output on day 7 for the functional analysis which typically employs destructive assays, unlike other assays employed in other work packages where outputs can be assessed on across multiple days via non-destructive media sampling.

An SOP was developed to detect enhanced mitochondrial ROS formation after cellular incubations with compounds/drugs (Report D8.1 SOP 4). Measurements were performed as described in the SOP which was initially developed on a 2D cell model (HepG2) and was expected to be applicable on spheroids, with only minor modifications. However, these methods required a significant amount of material and as such can be applied only to larger samples of material but are not compatible with small spheroids, as excessive numbers of spheroid would be needed per dose or time point. This increased requirement for greater spheroid numbers plus the already increased spheroid demand for other parameters in WP07 & WP08 meant it was not possible to apply this testing method widely across both 3D models. Focus continued on the Mitochondrial Function testing on both the cardiac and liver cell models.

RESULTS

Single Spheroid Measurement Consistency

A method was devised and optimised to facilitate single spheroid measurements using low volume glass capillaries. Data is presented illustrating the consistency of the measurement method. For both liver spheroids (Fig 1A & 1B) and cardiac spheroids (Fig 1C & 1D). Oxygen depletion profiles are very consistent with % CV values of < 8% for both methods, yielding Z' values of > 0.75. Validation was performed using the classical ETC inhibitor antimycin with testing immediately post treatment. Treatment caused inhibition of both liver and cardiac spheroids illustrating that the method can sensitivity detect ETC inhibition.

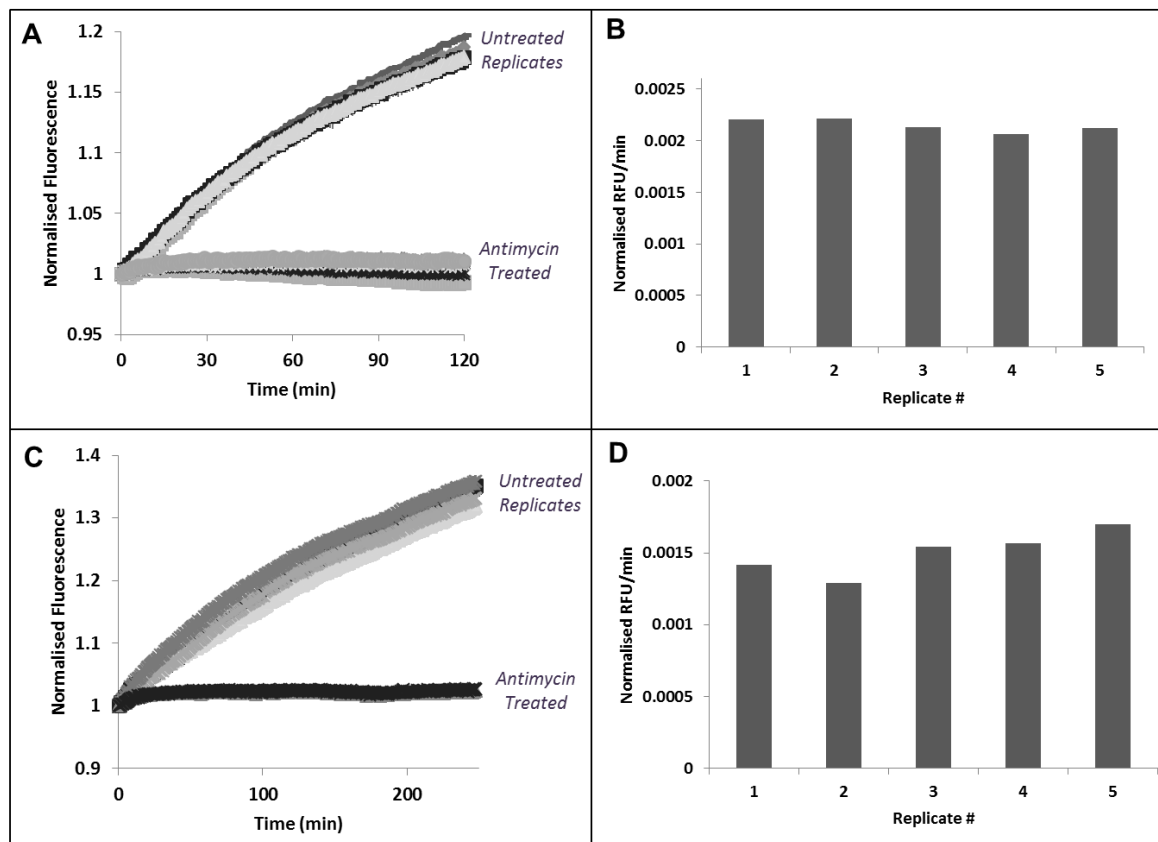


Fig 1: Method consistency in assessing liver (A&B) and cardiac (C&D) spheroid oxygen consumption.

Batch-to-batch consistency checks on cardiac spheroids revealed good consistency (Fig 2) but also illustrated that internal controls are necessary to facilitate data normalisation and comparison across measurement runs.

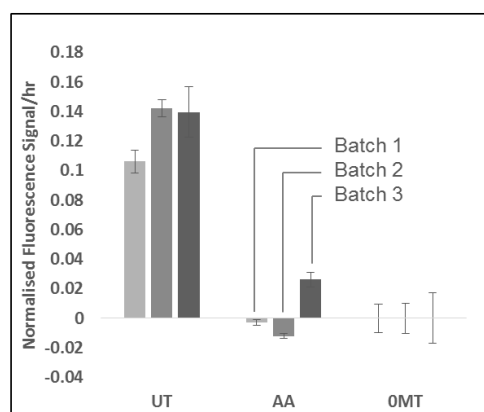


Fig 2: Cardiac spheroid batch consistency as measured by O₂ depletion

Cardiac Spheroid Mitochondrial Function measurements

Using this approach, the effect of the anthracycline Idarubicin on cardiac spheroid oxygen consumption was assessed across a relevant concentration range with parallel analysis of ATP content also performed. These data presented in Fig 3A illustrate that, 24 hours post treatment, no effect on ATP content or oxygen consumption was observed at treatment concentrations below 0.11 μ M, with higher

concentrations causing severe impairment of ETC activity and significant reduction in ATP content. In contrast, shorter exposure times reveal a divergence at 3 μM where significant inhibition of ETC activity is observed immediately post treatment, whereas ATP depletion has not yet occurred 2 hours post treatment, suggesting that ETC impairment precedes ATP depletion.

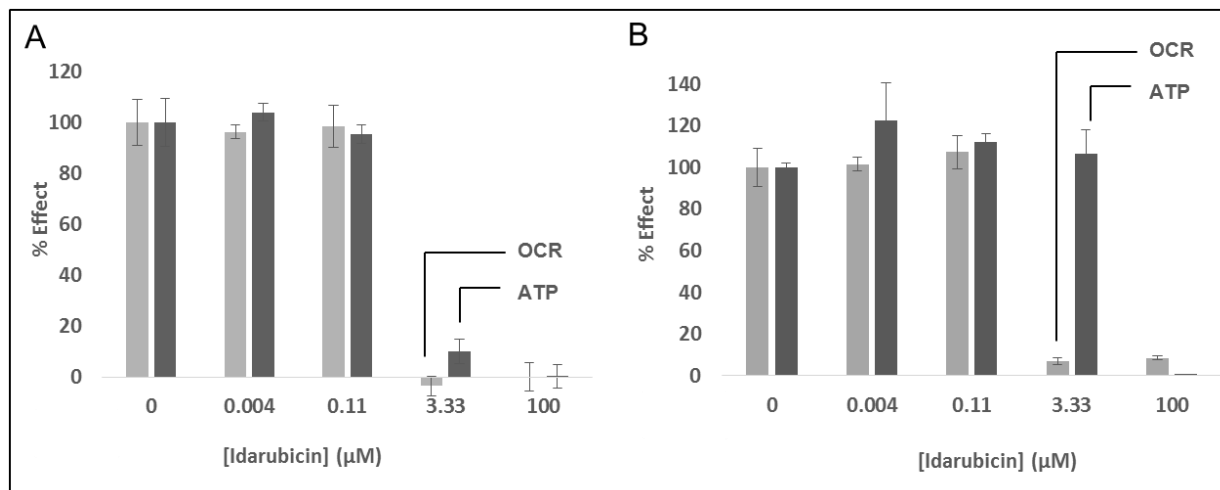


Fig 3: Effect of Idarubicin treatment on cardiac spheroid oxygen consumption and ATP content 24 hours post treatment (A) and 0-2 hours post treatment (B).

In an attempt to mimic *in vivo* exposures, a complex idarubicin dosing scheme was modelled and delivered from work package 4. Both ‘toxic’ and therapeutic’ conditions were modelled and exposure concentrations were varied as outlined in Fig 4 with O_2 consumption assessed 7 days post the initial treatment.

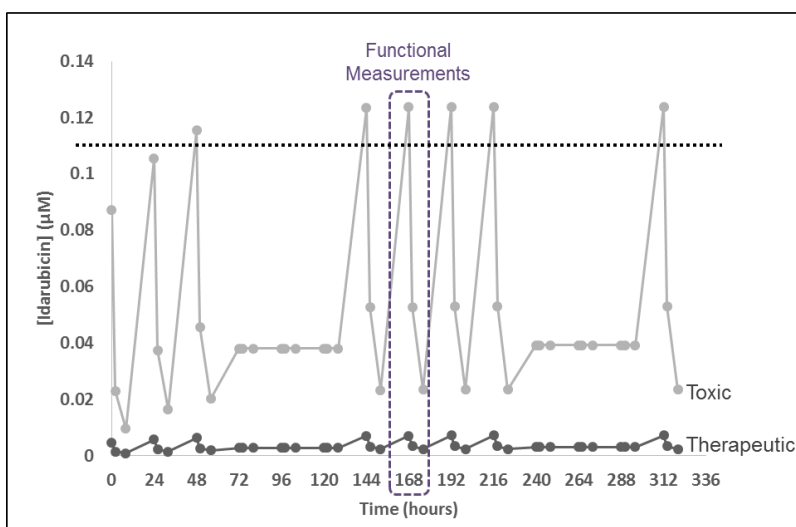


Fig 4: Graphical representation of 7 day long-term drug treatments of Idarubicin

Both O_2 consumption (Fig 5A) and ATP (Fig 5B) show minor reductions at the ‘therapeutic’ treatment condition with more severe reductions observed at the toxic treatment condition. Antimycin controls cause significant inhibition and both parameters.

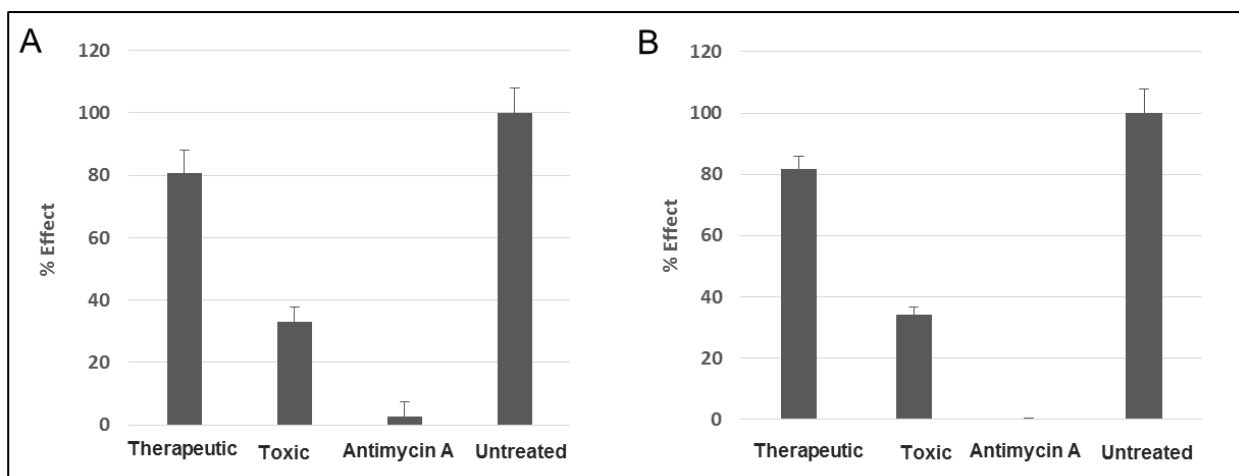


Fig 5: Effect of 7 day idarubicin treatment on O₂ consumption (A) and spheroid ATP content (B)

Once modelled and available from work package 4, and in an attempt to mimic *in vivo* exposures, a complex Doxorubicin and Epirubicin dosing scheme was applied to cardiac spheroids supplied by WP 4. Both 'toxic' and 'therapeutic' conditions were modelled and exposure concentrations were varied as outlined in Fig. 6 with O₂ consumption and ATP level assessed 7 days post the initial treatment.

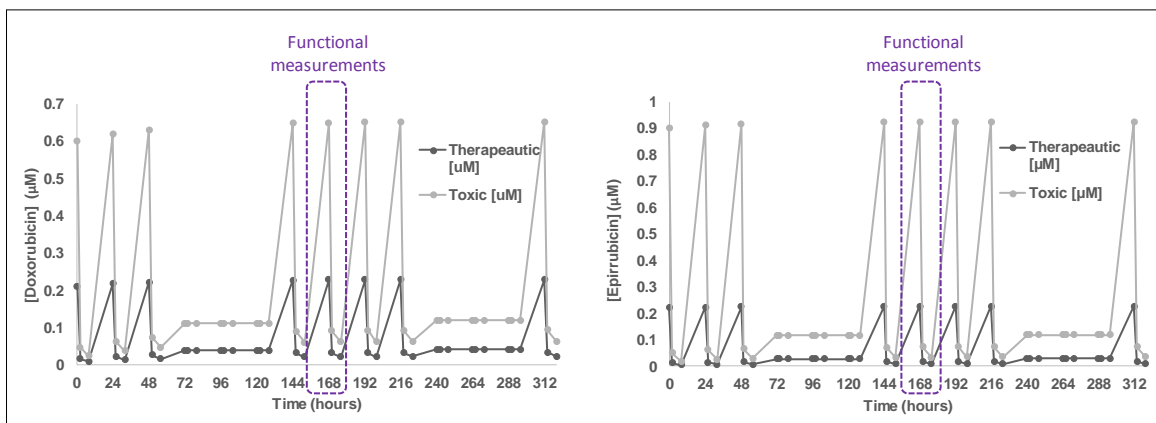


Figure 6: Graphical representation of 7 day long-term drug treatments of Doxorubicin & Epirubicin

Both O₂ consumption (Fig 7A) and ATP (Fig 7B) show minor reductions at the 'therapeutic' treatment condition with more severe reductions observed at the toxic treatment condition for Epirubicin especially. These data were submitted to work package 9 data warehousing during this period.

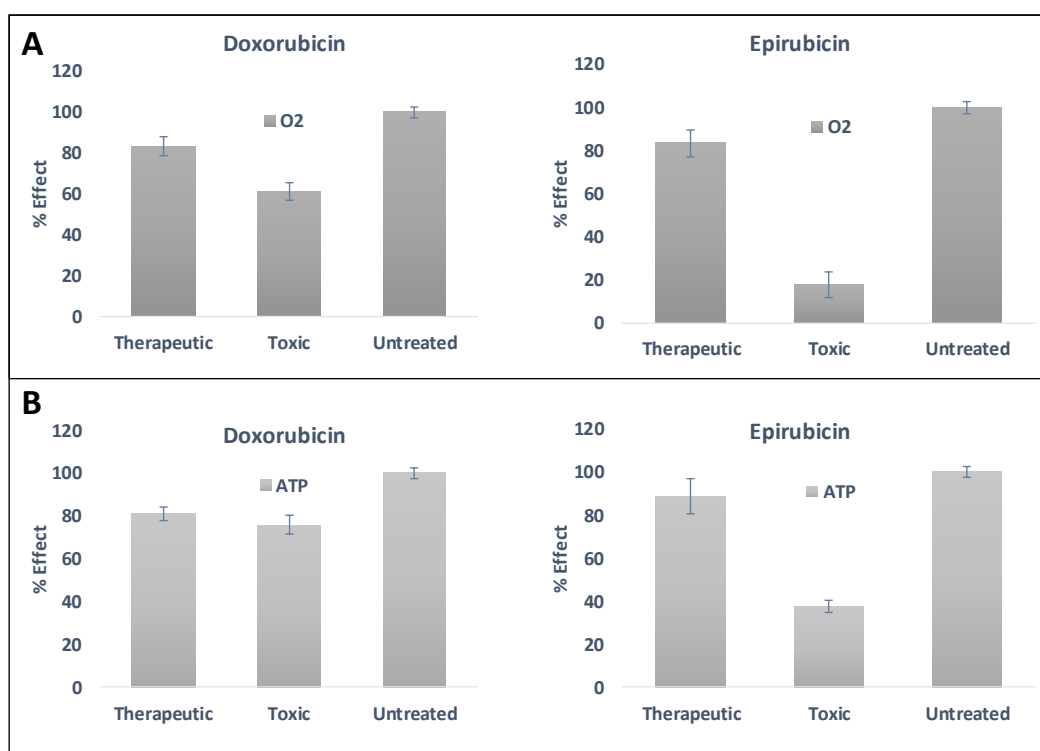


Figure 7: Effect of 7 day Doxorubicin & Epirubicin treatment on O₂ consumption (A) and spheroid ATP content (B).

Short time point exposure

To facilitate data interpretation and integration into the cardiac toxicity model, short time point mitochondrial function data for the 3 anthracyclines (Idarubicin, Doxorubicin and Epirubicin) was generated to compliment the long term 7 day treatment data. 'Toxic' and 'therapeutic' conditions for 0 - 2 hours dosing period of the complex dosing model supplied from WP4 were applied.

O₂ consumption (Fig. 8A) show minor reductions at the 'therapeutic' and 'toxic' treatment condition with more evident reduction observed at the toxic treatment condition for Epirubicin. ATP (Fig. 8B) show no real change from untreated levels at both the 'therapeutic' and 'toxic' treatment condition at this short time point. Antimycin controls cause almost complete inhibition as expected. These data were submitted to work package 9 data warehousing during this period.

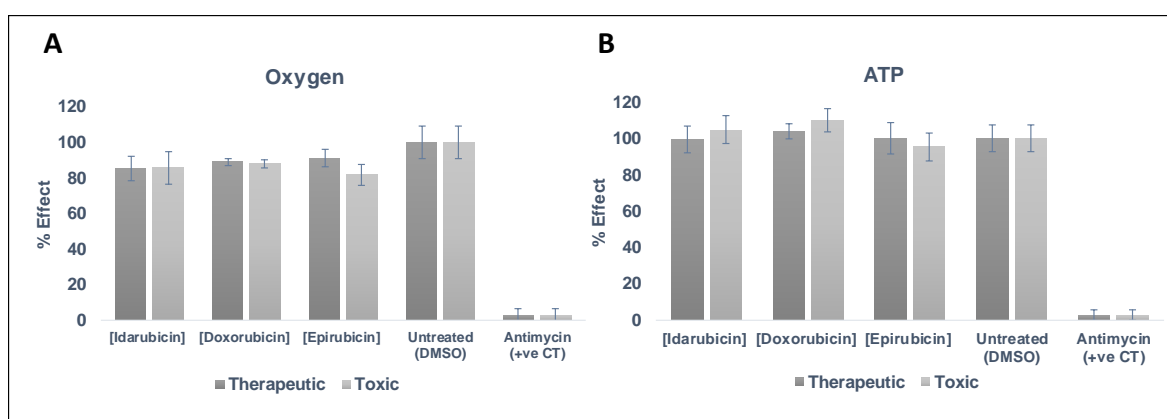


Figure 8: Effect of 2 hour Idarubicin, Doxorubicin & Epirubicin treatment on O₂ consumption (A) and spheroid ATP content (B)

A complex dosing scheme for 5-Fluororacile, Daunorubicin, Mitoxanthrone, Amiodarone, Docataxel and Paclitaxel was modelled and delivered from work package 4 during period 4. Both 'toxic' and 'therapeutic' conditions were modelled and exposure concentrations were varied as outlined in Fig 9 with O₂ consumption and ATP level assessed 7 days post the initial treatment.

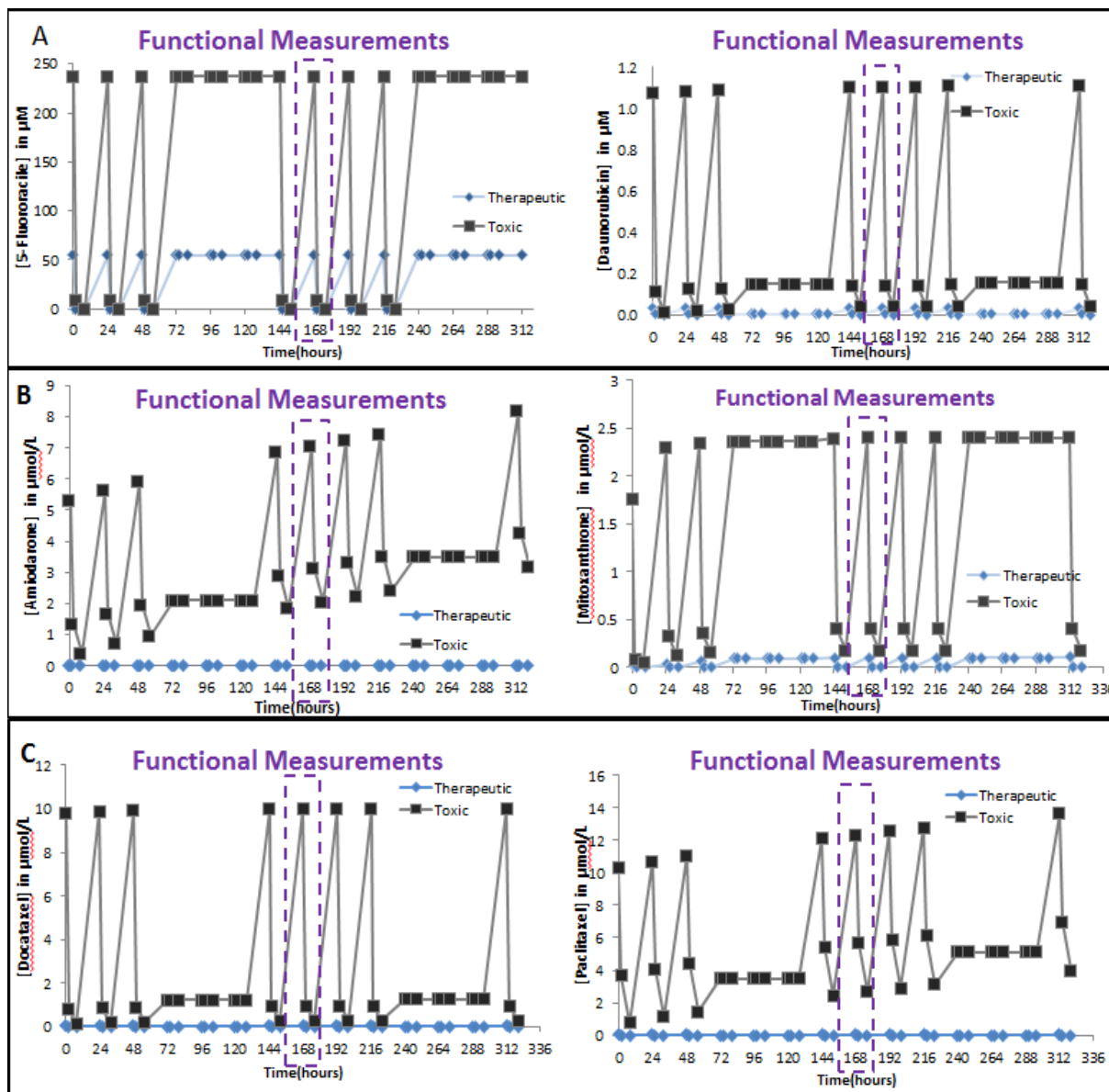


Fig. 9: Graphical representation of 7 day long-term drug treatments of **[A]** 5-Fluororacile (left) & Daunorubicin (right), **[B]** Amiodarone (left) & Mitoxanthrone (right) and **[C]** Docataxel (left) & Paclitaxel (right).

Both O₂ consumption (Fig 10A) and ATP measurement (Fig 10B) show minor reductions at the 'therapeutic' treatment condition for 5-Fluorouracile, Daunorubicin, and Mitoxanthrone with severe reductions (>90%) observed at the 'toxic' treatment condition for Daunorubicin and Mitoxanthrone compared to Untreated (vehicle treated) control. The 'toxic' treatment of 5-Fluorouracile on the other hand showed slight but minimal reduction below the 'therapeutic' treatment.

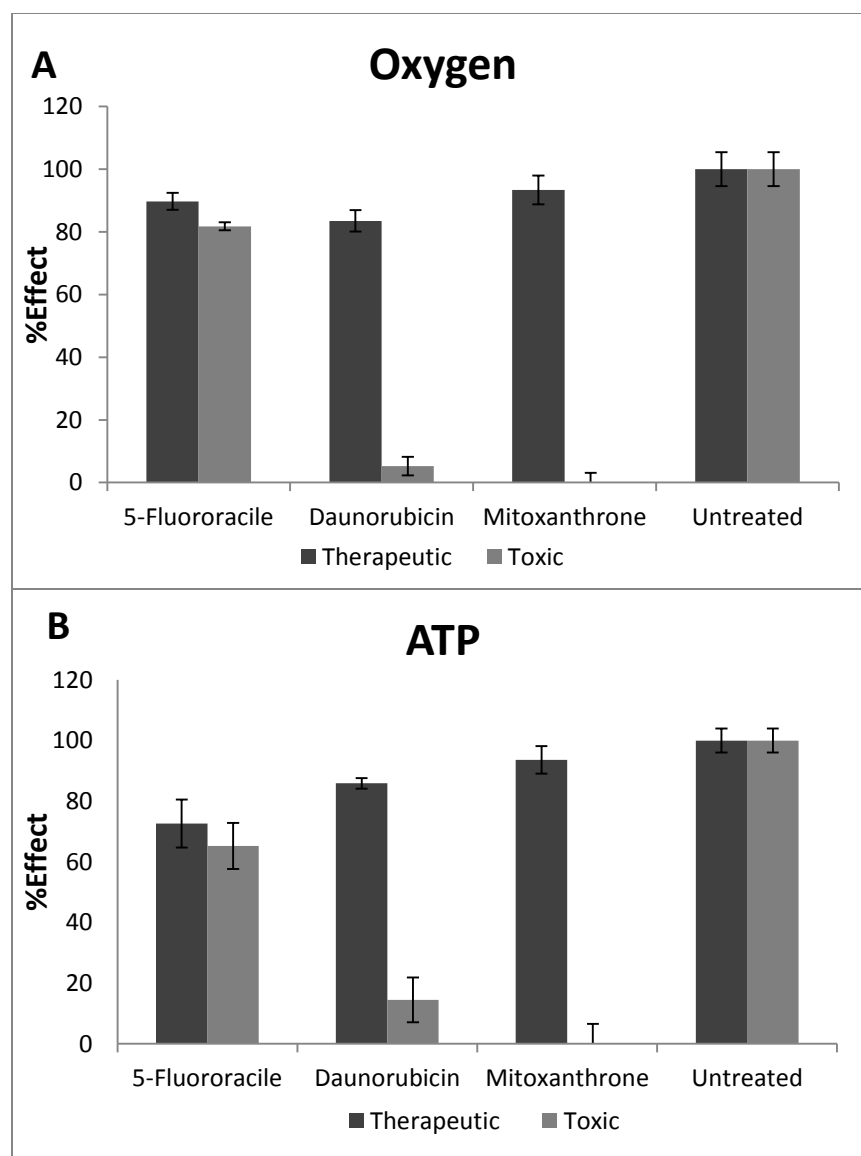


Fig. 10: Effect of 7 day treatments from 5-Fluorouracile, Daunorubicin, and Mitoxanthrone on **(A)** O₂ consumption and **(B)** spheroid ATP content.

Both 'therapeutic' and 'toxic' conditions for O₂ consumption (Fig 11A) of Amiodarone, Docataxel and Paclitaxel show very similar responses compared to Untreated (vehicle treated) control.

Very similar response is evident for ATP measurements (Fig 11B) at the 'therapeutic' and 'toxic' treatment, with a small increase in ATP level evident in 'toxic' Amiodarone treatment. The Antimycin A treatment works as expected inhibiting both O₂ consumption and ATP synthesis acting as a positive inhibitor control.

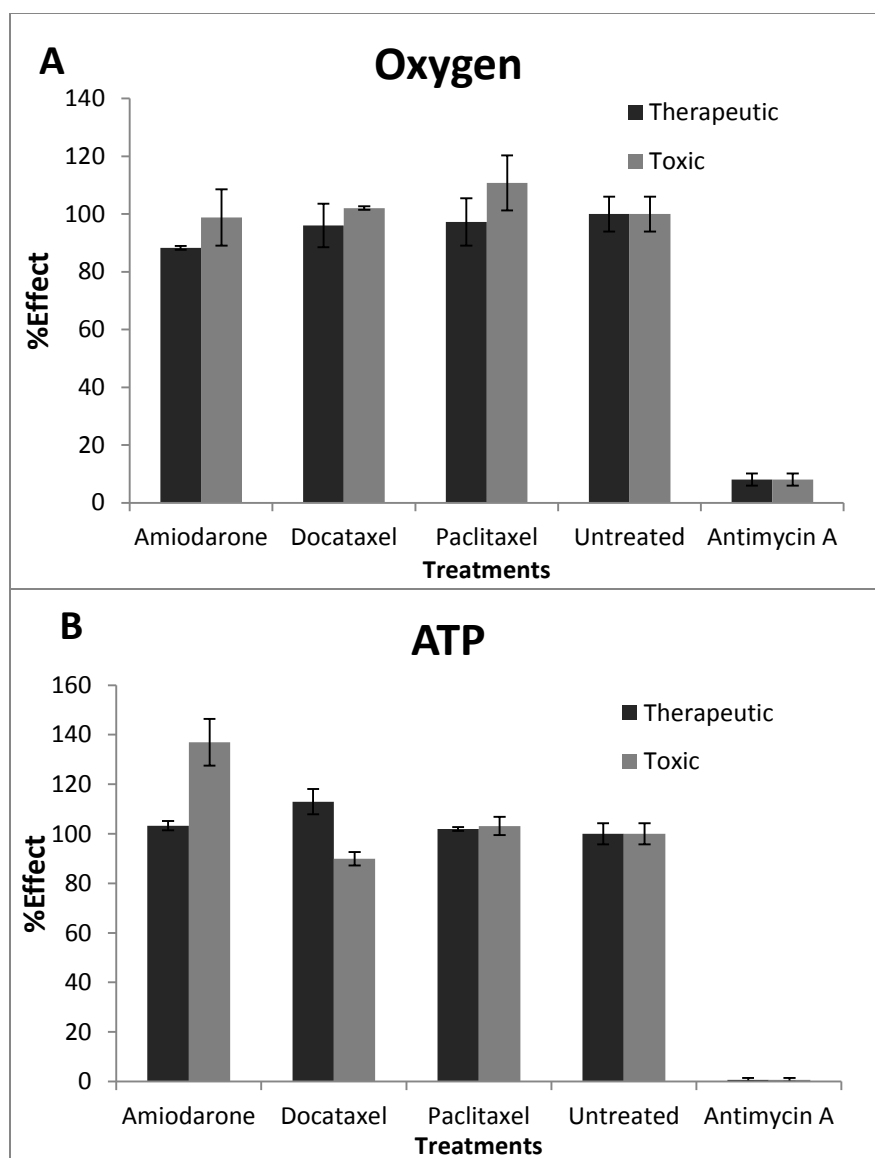


Fig. 11: Effect of 7 day treatments from Amiodarone, Docataxel and Paclitaxel on **(A)** O_2 consumption and **(B)** spheroid ATP content.

Liver Spheroid Mitochondrial Function Measurements

The single spheroid measurement approach was applied to all the available dosing schemes for the hepatic spheroid model in a similar manner. A complex Acetomenophen and Azathioprine dosing scheme delivered from work package 4 was applied, where both 'toxic' and therapeutic' conditions were modelled and exposure concentrations were varied as outlined in Fig 12 with O_2 consumption assessed 7 days post the initial treatment.

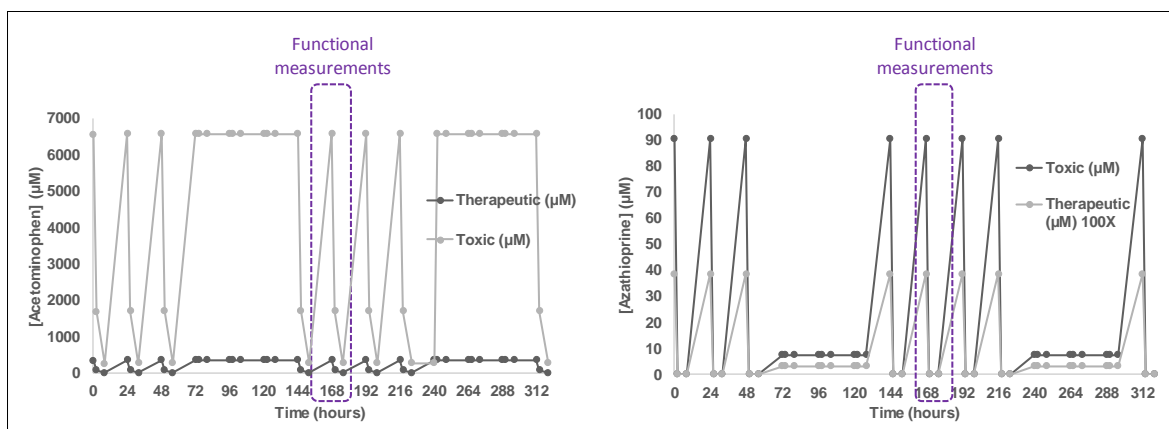


Figure 12: Graphical representation of 7 day long-term drug treatments of Acetaminophen and Azathioprine.

Both O_2 consumption (Fig 13A) and ATP (Fig 13B) show none if only minimal reductions at the 'therapeutic' treatment condition with more pronounced reductions observed at the 'toxic' treatment condition as expected from complex dosing model design (WP4). Antimycin controls cause significant inhibition as expected.

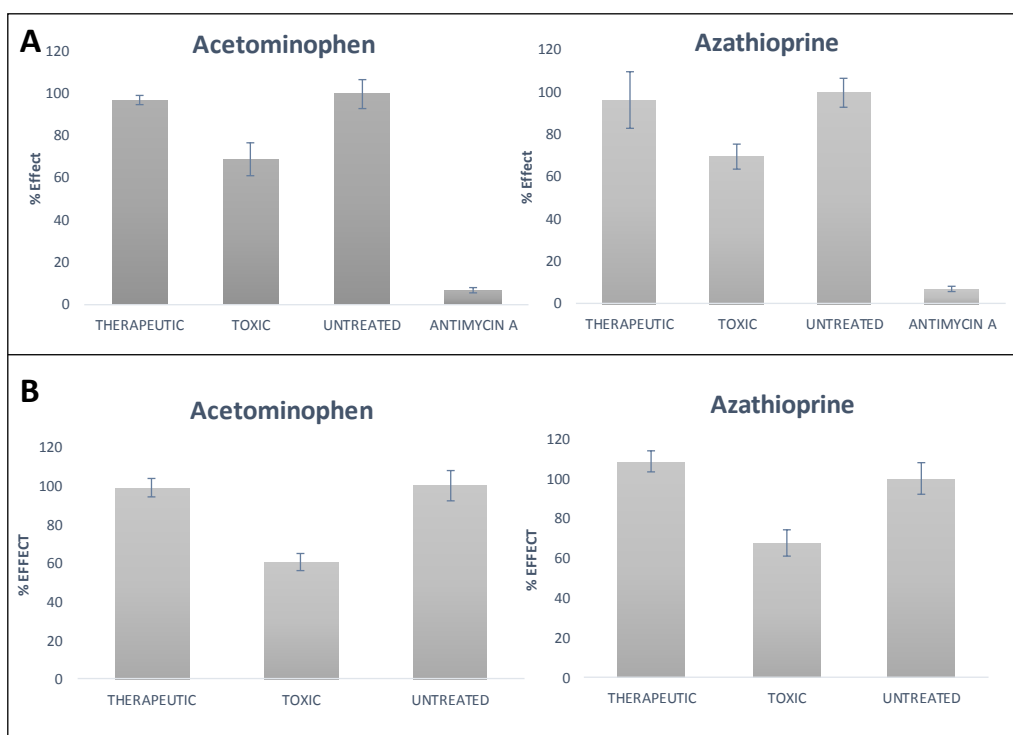


Figure 13: Effect of 7 day Acetaminophen and Azathioprine treatment on O_2 consumption (A) and spheroid ATP content (B).

The single spheroid measurement approach was again applied to the available dosing schemes for the hepatic spheroid model in a similar manner. A complex dosing scheme for 5-Fluorouracil and Phenytoin compounds delivered from work package 4 were applied, where both 'toxic' and 'therapeutic' conditions were modelled and exposure concentrations were varied as outlined in Fig 14 with O_2 consumption assessed 7 days (168 h) post the initial treatment.

The 7-day dosing scheme for phenytoin does not follow the typical dosing schemes observed previously and focuses on maintaining drug concentration at a similar level across the day rather than reducing it. The drug concentration for both the 'therapeutic' and 'toxic' treatment conditions remain almost constant with gradual concentration increase for the initial 72 hours and which is there maintained at a consistent concentration. The 'therapeutic' and 'toxic' concentrations only differ by a factor of 2 which differs quite a bit from other dosing schemes which is greater at > 4 or 5 fold.

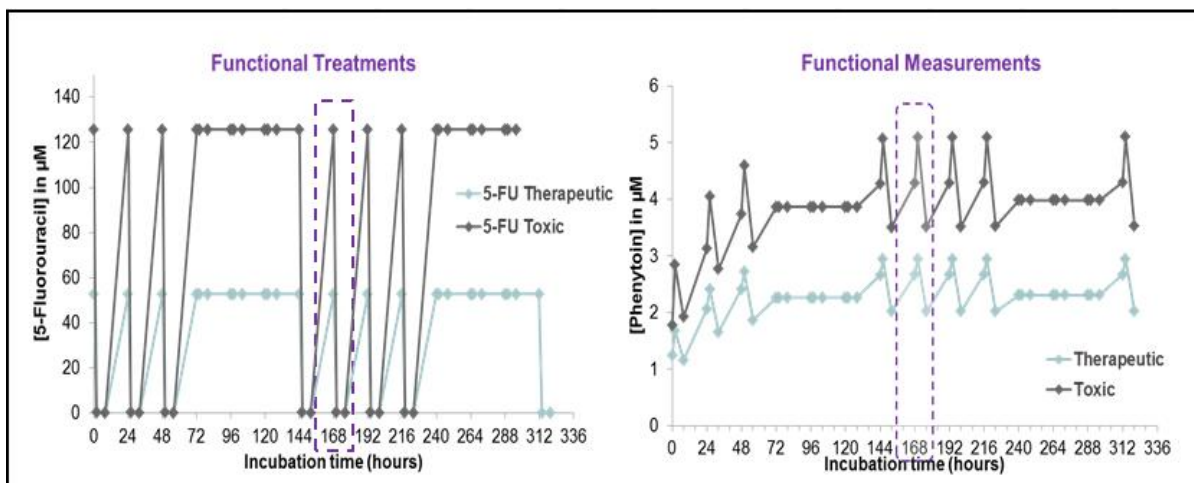


Fig. 14: Graphical representation of 7 day long-term drug treatments of 5-Fluororacil (left) and Phenytoin (right).

For 5-Fluororacil treated spheroids, both O_2 consumption (Fig 15A) and ATP (Fig 15B) show minimal reductions at the 'therapeutic' treatment condition with more pronounced reductions observed at the 'toxic' treatment condition. For Phenytoin treated spheroids, both O_2 consumption (Fig 15A) and ATP (Fig 15B) show a minimal changes in response compared to the vehicle treated samples for both the 'therapeutic' and 'toxic' treatment condition, with only slight, insignificant increase in O_2 consumption for therapeutic condition.

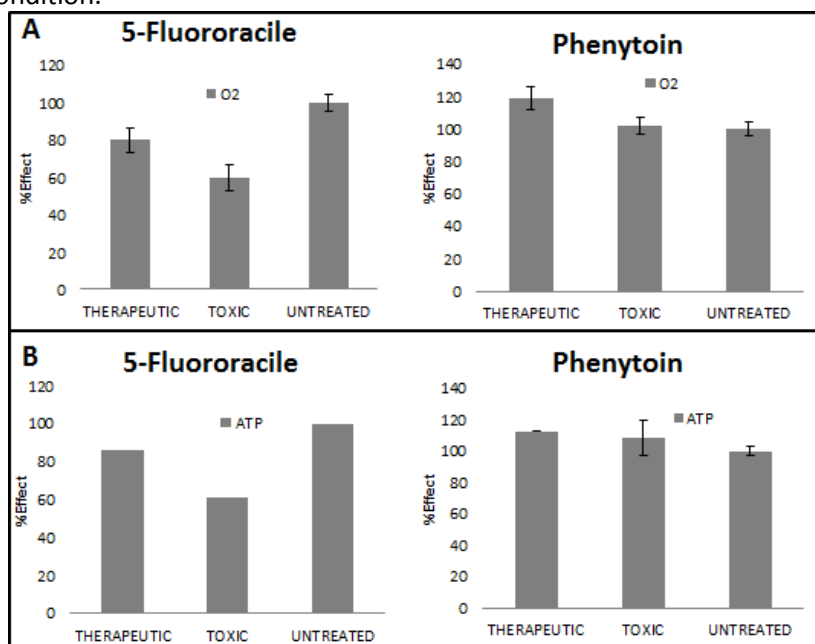


Fig 15: Effect of 7 day 5-Fluororacil and Phenytoin treatment on O_2 consumption (A) and spheroid ATP content (B).

Application of mitochondrial measurements on xCELLigence e-plates linked to T8.7 (Report D8.8)

Proof of concept data was also generated demonstrating the feasibility of measuring mitochondrial function on xCELLigence e-plates (Fig. 16) thereby facilitating sequential analysis of cardiomyocyte mitochondrial function and beating with a view to providing a more holistic picture of the impact of drug treatment on cardiomyocyte function.

This was further advanced where assessment of both glycolytic flux (pH-Xtra) and intracellular oxygen (MitoXpress-Intra) measurements on the xCELLigence E-plates has been optimised and demonstrated showing combined analysis with arrhythmia measurements in T8.7 are possible. This further facilitates the sequential combined analysis of cardiomyocyte mitochondrial function and metabolism, with beating, allowing the assessment of drug induced altered beat rate and subsequent change on metabolism and mitochondrial function.

Cardiomyocyte beating was assessed on 96 well E-plates using the xCELLigence Cardio system (ACEA), (See D8.8 Deliverable Report). Cell metabolism was measured on the same plate using multiplexed fluorometric measurements of O₂ consumption (MitoXpress-Xtra), glycolytic flux (pH-Xtra) and cellular oxygenation (MitoXpress-Intra).

Classical Mitochondrial Compounds were tested initially to assess assay performance, as well as known Cardio-active drugs such as Isoproterenol, Nifedipine and E-4031, to assess specific cardiomyocyte responses.

Assessing Assay performance on E-Plates

Antimycin and Rotenone treatment causes an immediate inhibition of mitochondrial function while FCCP treatment causes an immediate uncoupling of OxPhos (Fig. 16). A clear concentration dependent decrease in O₂ consumption due to reduced ETC activity and an accompanying increase in ECA was measured (Fig. 17), while ATP level remain stable, suggesting that ATP depletion is ameliorated through increased glycolytic flux. Cardiomyocyte beating continues in the presence of inhibitory concentrations of Antimycin, Rotenone and FCCP and is maintained for over 24h, as seen in D8.8 deliverable report. This suggests that increased glycolytic flux supplies sufficient ATP to facilitate cardiomyocyte beating despite complete impairment of OxPhos.

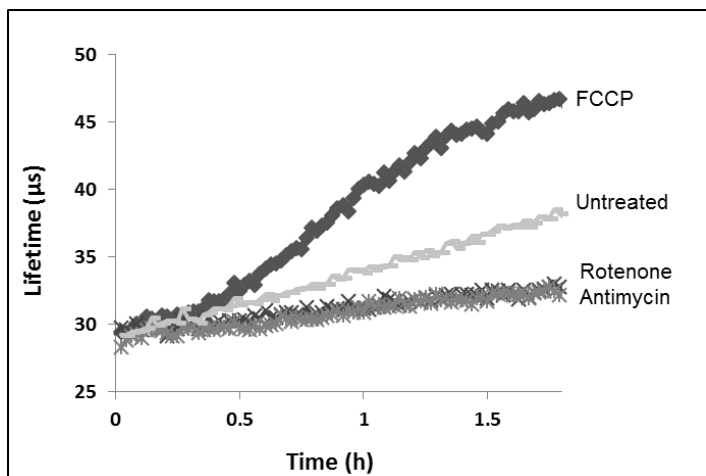


Fig 16: MitoXpress-Xtra based oxygens consumption measurements performed on xCELLigence E-plates measured on a TRF plate reader.

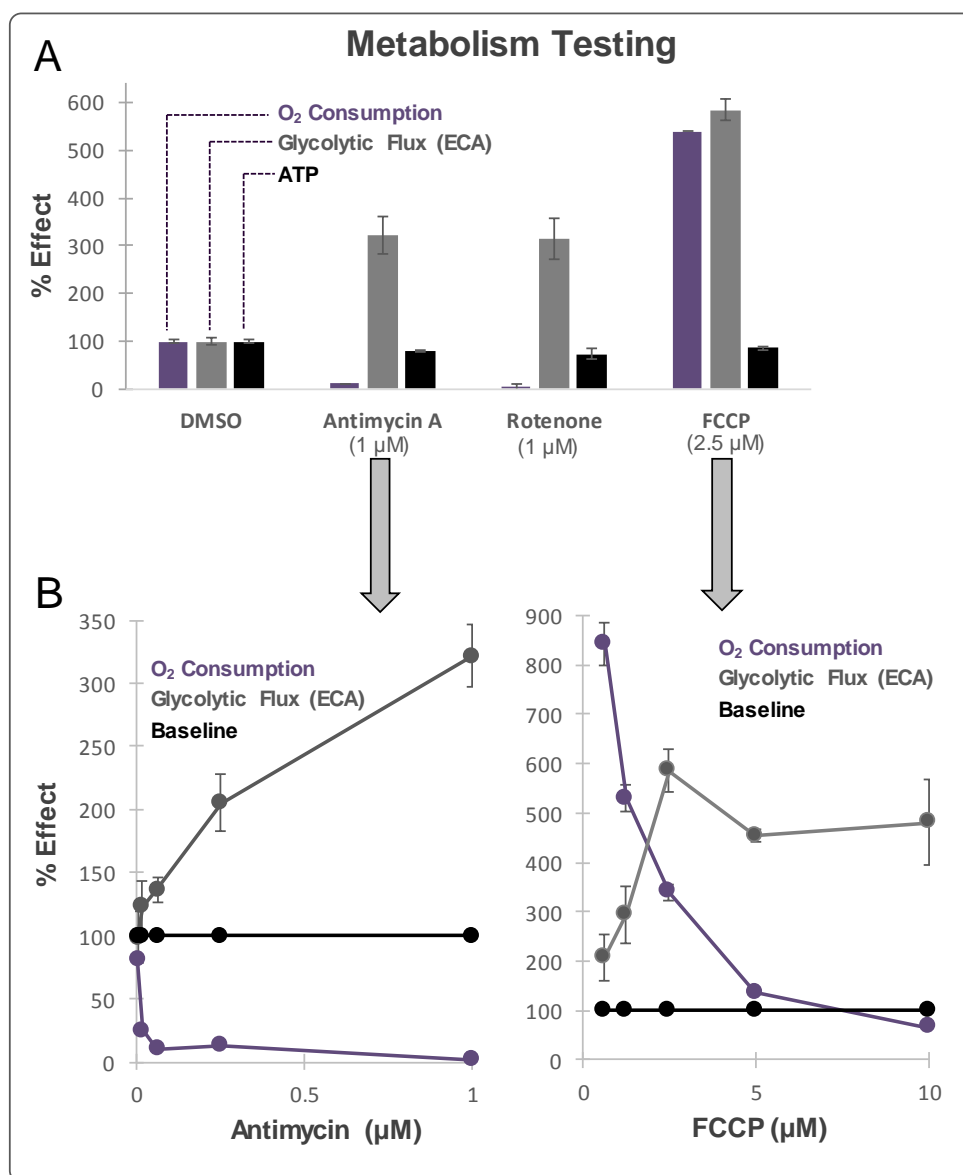


Figure 17: Oxygen Consumption and Glycolytic Flux metabolism measurements of antimycin, rotenone and FCCP treated cardiomyocytes, performed on xCELLigence E-plates for single concentrations versus ATP level (A) and dose response of antimycin and FCCP treatments (B) .

Assessing Cardiomyocyte Metabolic Response to Cardio-active Drugs on E-Plates

Treatment of (β -adrenoreceptor agonist) isoproterenol causes an increase in cardiomyocyte beat rate Fig 18A, and in D8.8 deliverable report. An immediate increase in O₂ consumption suggesting increased aerobic ATP production in response to increased ATP demand Fig 18B. ECA is not increased significantly (data not shown) suggesting that OxPhos rather than glycolysis is supplying the additional ATP requirements.

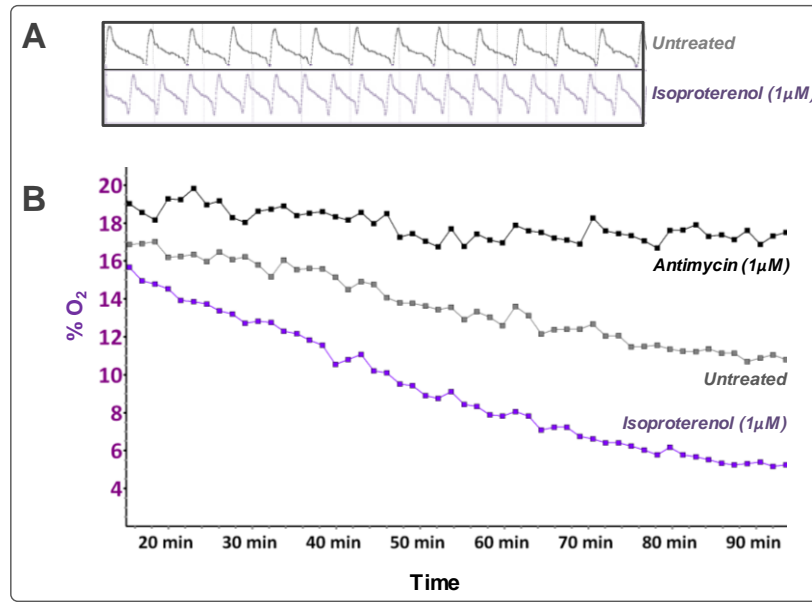


Figure 18: Impact of isoproterenol treatment on cardiomyocyte beat rate (A) and metabolism (O₂ consumption) (B) measured on a FLUOstar Omega (BMG Labtech). Increased oxygen consumption due to increased beat rate caused a more rapid oxygen depletion compared to untreated. Antimycin control caused expected decreased O₂ consumption as expected.

Treatment with L-type Ca²⁺ channel antagonist nifedipine, decreases cardiomyocyte beating. Fig.19A illustrates the dose dependant decrease in cardiomyocyte O₂ consumption. ECA is also reduced (not shown). Fig 19B summarises the effect the hERG channel inhibitor E-4031 cardiomyocyte metabolism, where significant reductions in O₂ consumption are also observed with minor reduction in ECA. The corresponding beat rate data for both nifedipine and E-4031 treatment is shown in D8.8 report. Beating is perturbed (measured at 30 min).

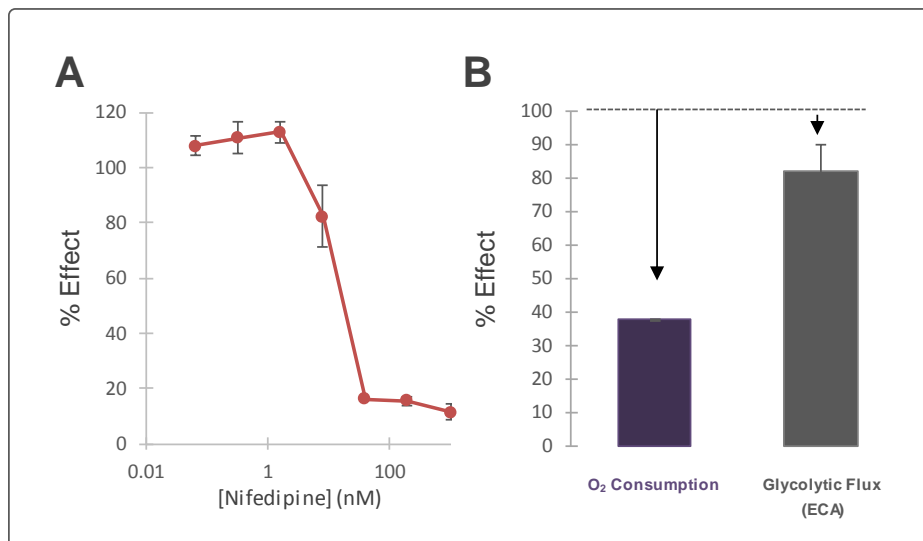


Figure 19: Impact of Nifedipine dose response on O₂ consumption (A) and E-4031 (B) on metabolism (O₂ consumption and ECA) Metabolism data presented as percentage of untreated control. Corresponding beat rate data in D8.8 report (beating measured 30 min post-treatment) for T8.7.

Impact of Increased Beat Rate on Cellular Oxygenation

MitoXpress-Intra intracellular O₂ probe measurement of cellular oxygenation shown in Fig 20 shows the effect of pharmacologically altering cardiomyocyte beat rate on cardiomyocyte oxygenation. Basal O₂ concentrations are at ~14%, (reduced from ambient (~21%)). ETC inhibitor Antimycin treatment causing intracellular O₂ levels to return to ambient levels, whereas β -adrenoreceptor agonist isoproterenol causes increased cardiomyocyte beat rate which in turn causes an increase in oxygen consumption (Fig 20). This causes a significant but temporary reduction in O₂ availability with values of ~6% observed for >15 min despite cells being cultured and measured at 21% O₂.

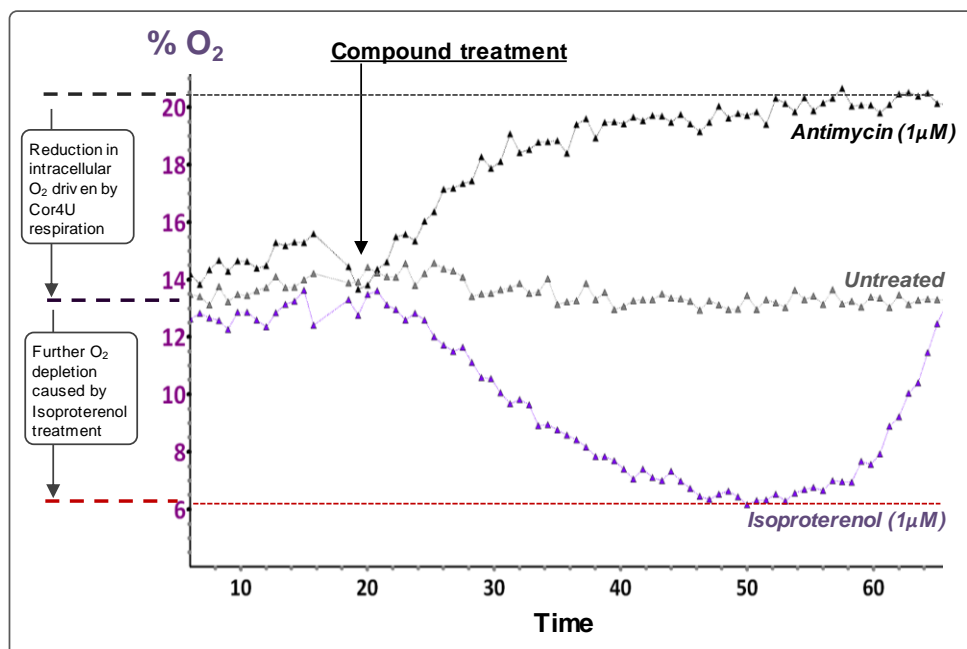


Figure 20: Effect of pharmacologically altering iPS derived cardiomyocyte beat rate on cardiomyocyte oxygenation. Intracellular oxygen measurements performed on xCELLigence E-plates measured on a TR-F plate reader.

REFERENCES

C. Carey, C. Bertinetti-Lapatki, A. Roth, J. Hynes. Assessing the impact of drug treatment on cardiomyocyte function through combined analysis of beating, metabolic flux and cellular oxygenation. Society of Toxicology Annual Meeting, 2016.

DIFFICULTIES

- Significant difficulties arose around access to patient samples for *ex vivo* mitochondrial analysis (oxygen consumption & ESR). This emerged as an issue due the fact that the amount of material required to conduct all analyses across WP7 and WP8 exceeded the amount of material available *via* needle biopsy. Amount of material was not sufficient to facilitate all of the *ex vivo* analysis planned. The 'omics measurements were prioritised to ensure maximum data return to the project, meaning that no functional mitochondrial analysis (Oxygen consumption or ESR), were performed for *ex vivo*, patient samples. Instead it was determined that information on mitochondrial function and caspase activity would best be attained, considering the limitation in biomaterial, through assessing

mitochondrial and caspase protein expression and metabolite patterns through proteomic and metabolomic analysis respectively;

- ESR measurements of both the 3D cell models for T8.4 could not be applied due the high number of Spheroids required for single measurements.