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Collaborative project

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**Deliverable Report D8.6:**

**Data set describing the impact of drug treatment on the apoptosis induction  
of both 3D models**

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Work package 8

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**Maastricht University (UM)**

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<b>PP</b>	Restricted to other programme participants (including the Commission Services)	
<b>RE</b>	Restricted to a group specified by the consortium (including the Commission Services)	
<b>CO</b>	Confidential, only for members of the consortium (including the Commission Services)	

### Contributions to deliverable - Internal review procedure

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### Contents

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## PUBLISHABLE SUMMARY

Data set describing the impact of drug treatment on the apoptosis induction of both 3D models.

## OBJECTIVES

To generate a data set describing the impact of drug treatment on the apoptosis induction of both 3D models, using Caspase 3/7 fluorimetric assay.

## INTRODUCTION

Caspases are activated in response to a wide range of cell death stimuli and catalyse the disassembly of the cell through controlled proteolysis of up to 400 cellular substrates. Here, the effector caspases 3 and 7 are measured as an indicator of apoptotic signalling. These heterodimeric cysteine proteases cleave polypeptide substrates at the C-terminal side of an aspartate residue within a preferred tetra-peptide sequence of aspartate-glutamate-valine-aspartate (DEVD). Cleavage of a luminogenic reporter containing a DEVD sequence (Promega) causes the release of aminoluciferin, with luminescence therefore indicative of caspase activity.

The protocol used was previously optimised for the measurement of caspase activity with HCT116 spheroids, however additional optimisation was required to generate robust caspase activity data from the spheroids tested here (organotypic spheroid co-culture of primary human hepatocytes and kupffer cells, and cardiac spheroids generated using iPS derived cardiomyocytes). The spheroids were delivered from Partner InSphero via Work package 5.

In this report, data on 9 cardiac drug treatments (Idarubicin, Doxorubicin, Epirubicin, Daunorubicin, 5-fluorouracil, Amiodarone, MitoXanthrone, Docetaxel, 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-fluorouracil, Phenytoin) using available dosing schemes of the hepatic spheroid model are also presented. At time of reporting, one other cardiac drug (Celecoxib) and a further three liver drugs (Isoniazid, Valproic acid, and Cyclosporine) are being tested. A further 3 liver drug treatments are planned to be tested by both WP05 and WP08 after month 48 to ensure 10 drugs of both cardiac and liver are completed.

To improve performance, the caspase assay was re-optimised assessing exposure time, shake times, wash steps and positive controls.

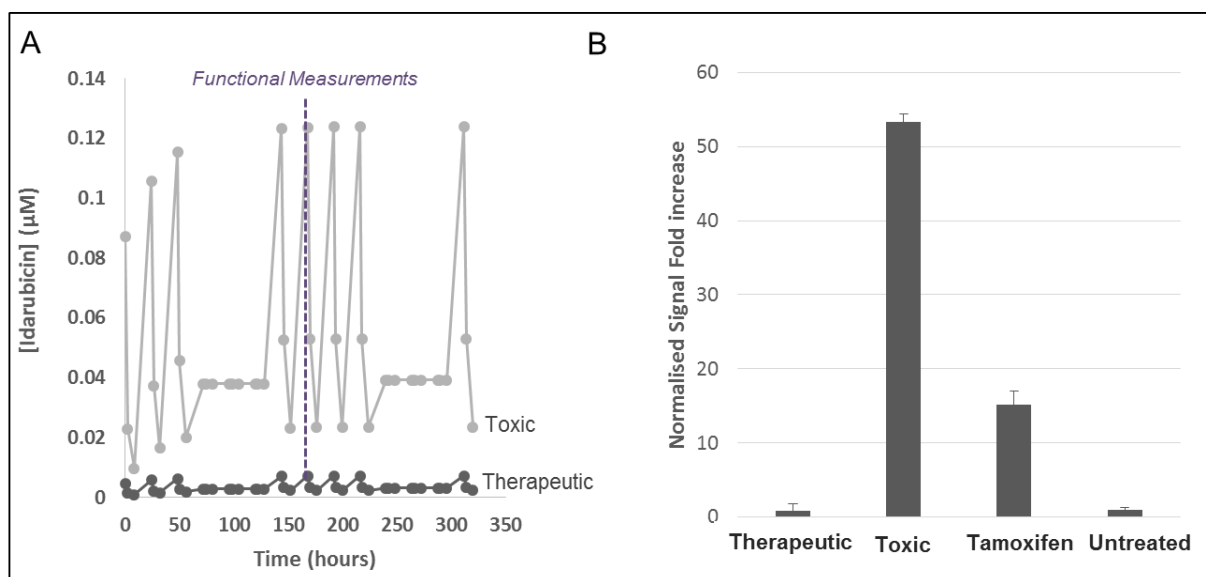
## RESULTS

### Additional Caspase Assay optimisation

Further assay optimisation for spheroid measurements was employed in order to further improve assay performance ahead of proper assessment of drug tested spheroids. It was re-optimised assessing exposure time, shake times, wash steps and positive controls.

## Cardiac Spheroid Caspase Activity

Using this re-optimised assay the ability of the anthracycline Idarubicin to activate caspase activity in cardiac spheroids was assessed across a relevant concentration range. Using a reoptimised assay the ability of the anthracycline Idarubicin to activate caspase activity in cardiac spheroids was assessed across a relevant concentration range. 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. 1A with caspase assessed 7 days post the initial treatment, as summarised in Fig. 1A. These data illustrate that the ‘toxic’ exposure induced significant caspase activity, with activity also observed for the positive control tamoxifen, while the therapeutic Idarubicin treatment showed no detectable caspase activity on day seven of the dosing scheme.

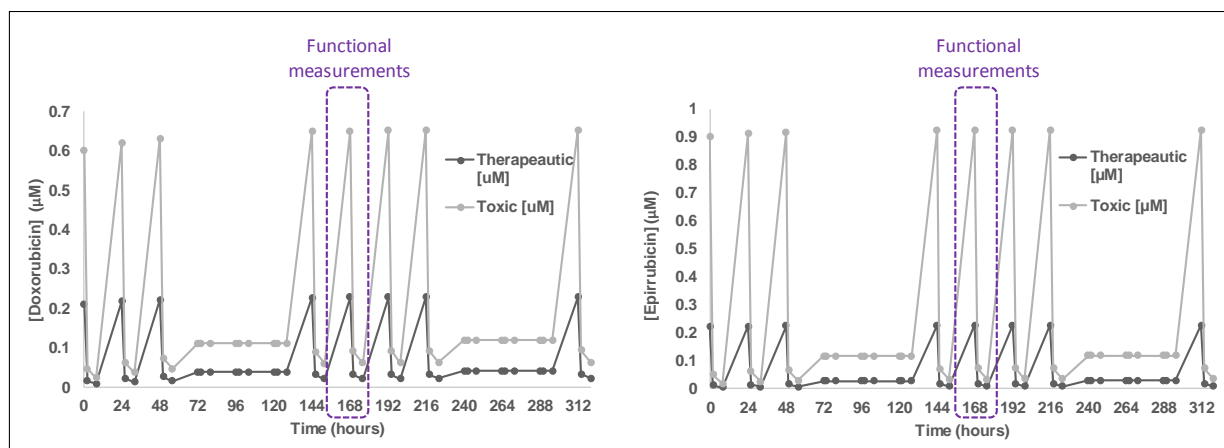


**Fig 1:** Idarubicin drug treatment schemes for both ‘toxic’ and ‘therapeutic’ conditions (A) and the caspase activity resulting from these treatments as measured 7 days post initial treatment (B).

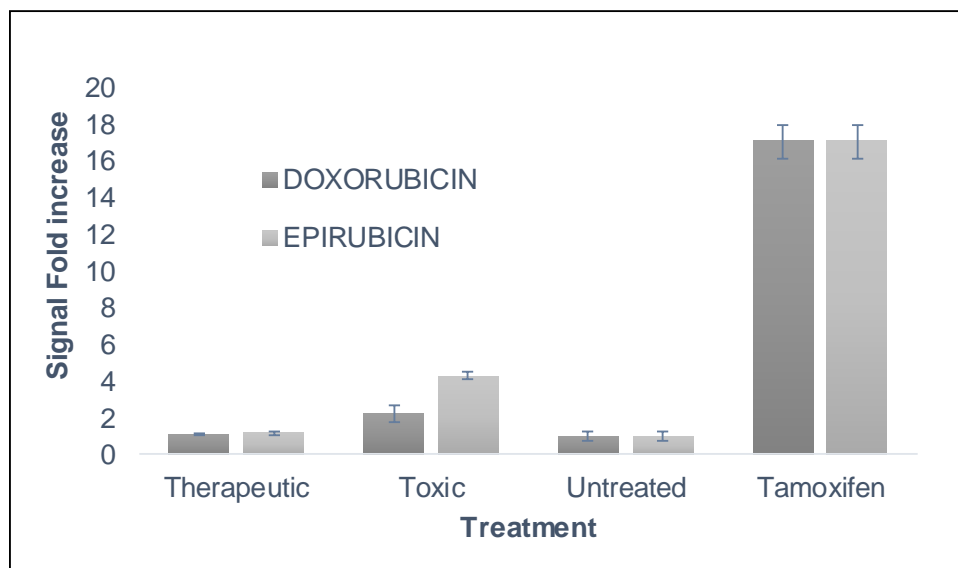
Using the same re-optimised assay applied to Idarubicin testing above, the ability of the anthracyclines Doxorubicin and Epirubicin to activate caspase activity in cardiac spheroids was assessed across a relevant concentration range for both ‘therapeutic’ and ‘toxic’ concentrations. In an attempt to mimic *in vivo* exposures, a complex Doxorubicin and Epirubicin 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. 2, with caspase assessed 7 days post the initial treatment, as summarised in Fig 3. These data illustrate that both the ‘toxic’ and ‘therapeutic’ exposure for both Doxorubicin and Epirubicin did not induce strong caspase activity on day seven of the dosing scheme, while significantly strong activity was observed for the positive control tamoxifen.

Epirubicin treatment did show minimal increased caspase activity compared to Untreated, particularly the ‘toxic’ condition.



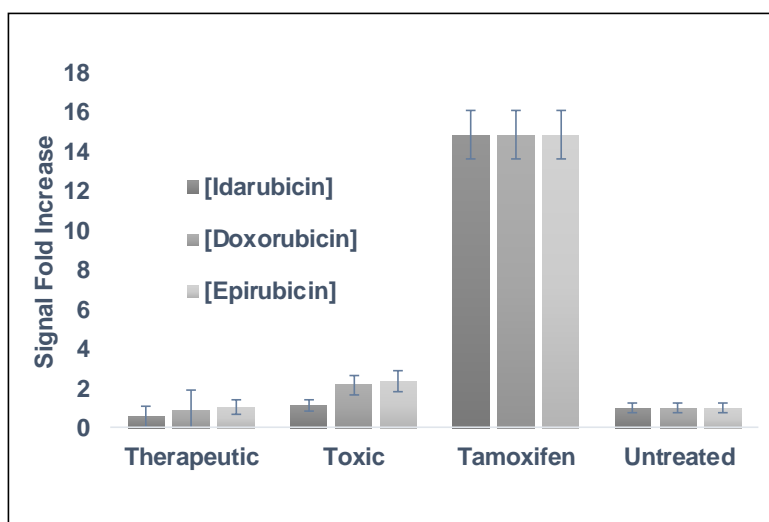
**Fig 2:** Graphical representation of 7 day long-term drug treatments of Doxorubicin & Epirubicin



**Fig 3:** Caspase activity resulting from Doxorubicin and Epirubicin treatment measured 7 days post initial treatment, using the complex dosing schemes for both 'toxic' and 'therapeutic' conditions.

### Short time point exposure

To facilitate data interpretation and integration into the cardiac toxicity model short time point caspase activity data for the 3 anthracyclines (Idarubicin, Doxorubicin and Epirubicin) was generated (Fig 4), in parallel with the oxygen consumption and ATP measurements. 'Toxic' and 'therapeutic' conditions for 0 - 2 hours dosing period of the complex dosing model supplied from WP04 were applied.

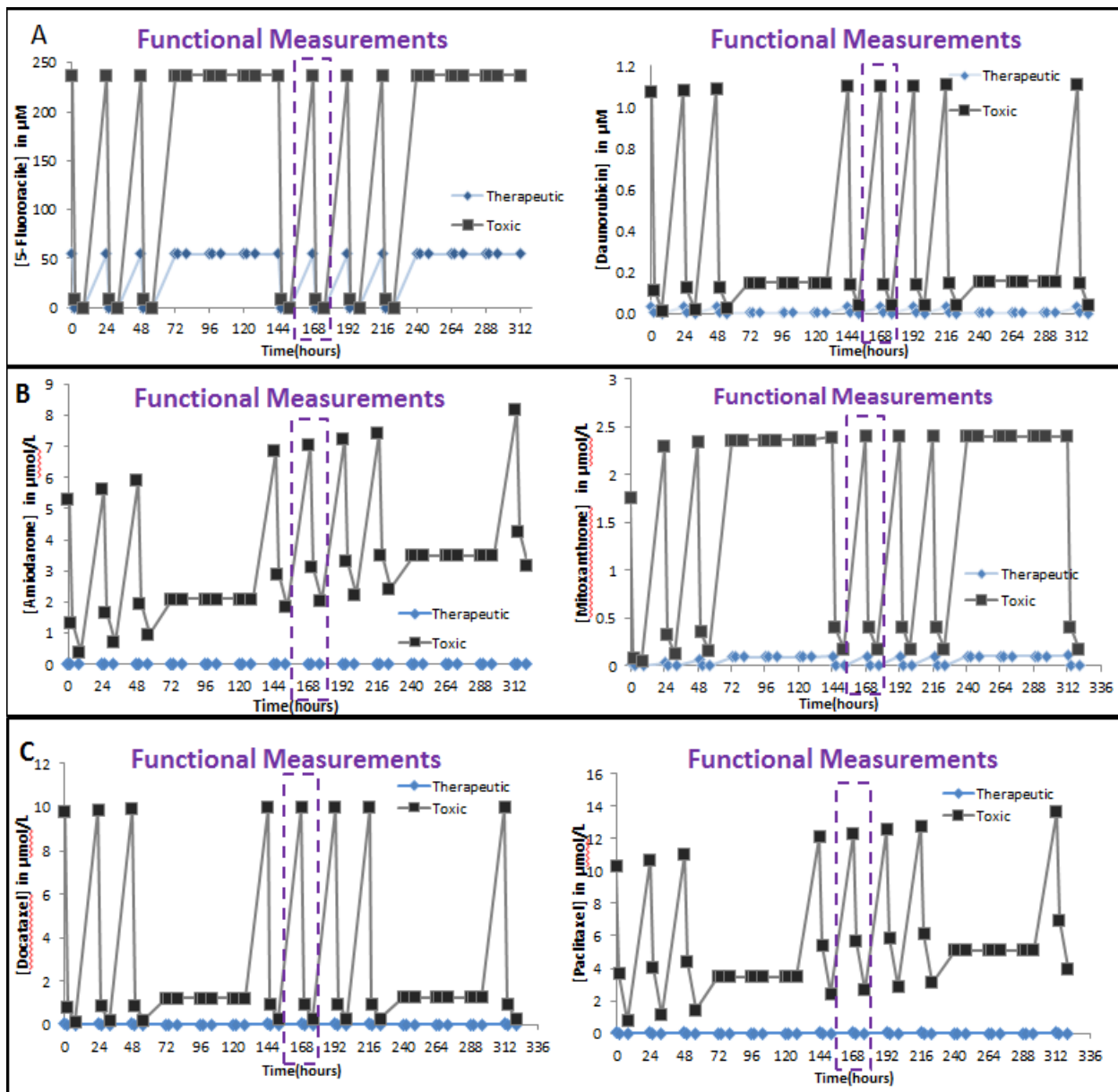


**Fig 4:** Caspase activity resulting from Idarubicin, Doxorubicin and Epirubicin treatment measured 2 hours post initial treatment, using the complex dosing schemes for both 'toxic' and 'therapeutic' conditions.

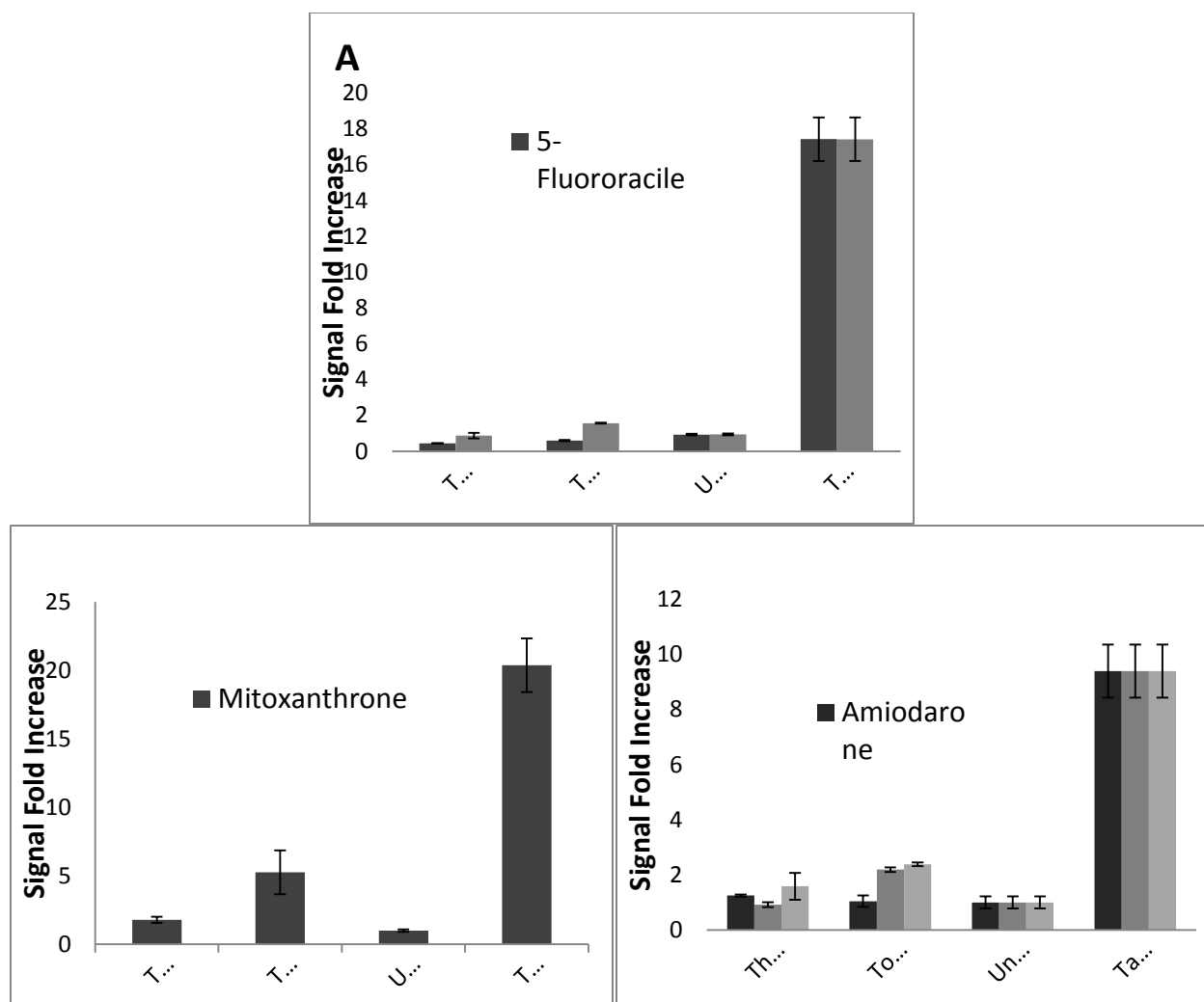
Again using the re-optimised assay, the ability of 6 further cardiac drugs (5-Fluorouracil, Daunorubicin, Mitoxantrone, Amiodarone, Docetaxel and Paclitaxel) to activate caspase activity in cardiac spheroids was assessed across a relevant concentration range for both 'therapeutic' and 'toxic' concentrations. In an attempt to mimic in vivo exposures, a complex dosing scheme for the above mentioned compounds was modelled and delivered from Work package 4. Both 'toxic' and 'therapeutic' conditions were modelled and exposure concentrations were varied as outlined in Fig. 05, with caspase activity assessed 7 days post the initial treatment, as summarised in Fig 6.

These data illustrate that both the 'toxic' and 'therapeutic' exposure for the abovementioned compounds did not induce strong caspase activity as measured on day seven of the dosing scheme, while significantly strong activity was observed for the positive control tamoxifen, measured 24 h post treatment (50  $\mu$ M). Mitoxantrone 'toxic' treatment did show an increased caspase activity compared to Untreated condition, but not to the high level detected in the Tamoxifen positive control.

Daunorubicin, Docetaxel and Paclitaxel treatments in the 'toxic' condition resulted in a slightly elevated caspase activity compared to the untreated, but again not to the high level detected in the Tamoxifen positive control exhibiting strong caspase 3/7 activity. No significant signal fold increase (caspase activity) was detected in the 2 remaining compounds 5-FL, and Amiodarone 7 days post treatment in either the 'toxic' or 'therapeutic' condition.



**Fig. 5:** 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).

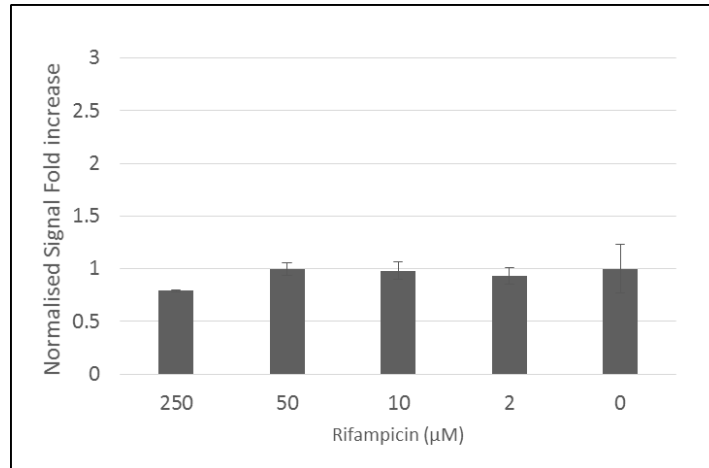


**Fig. 6:** Caspase activity resulting from [A] 5-Fluororacile, Daunorubicin, [B] Mitoxanthrone, and [C] Amiodarone, Docataxel, and paclitaxel treatment measured 7 days post initial treatment, using the complex dosing schemes for both 'toxic' and 'therapeutic' conditions

### Liver Spheroid Caspase Activity

In preparation for similar drug treatment of liver spheroids, a rifampicin dose response as assessed 24h post-treatment. Data are presented in Fig. 2 and show no detectable caspase activity across the concentrations tested. These data are consistent with ATP depletion assessments and will facilitate further optimisation of the rifampicin dosing model being supplied from WP04.

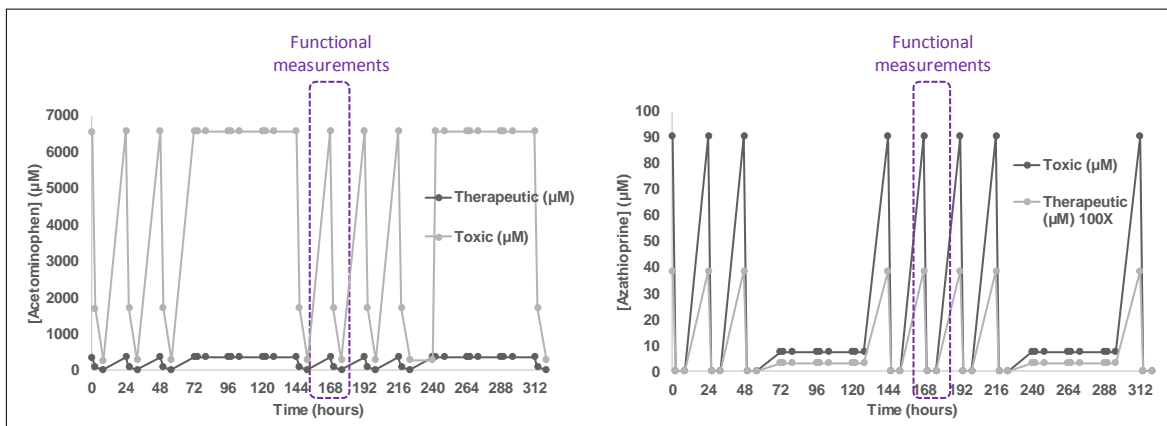




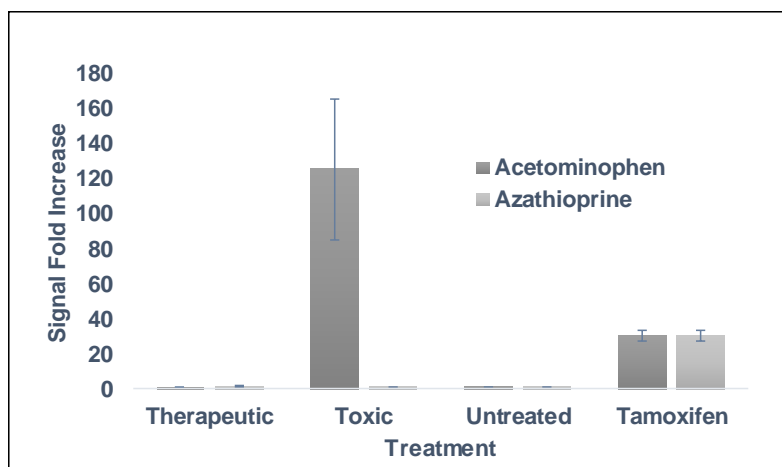
**Fig 7:** Measured caspase activity 24h post rifampicin treatment.

Complex dosing schemes delivered from Work package 4 for Acetaminophen and Azathioprine (Fig. 8) were applied to liver spheroids, where the ability of the compounds to activate caspase activity in liver spheroids was assessed 7 days post initial treatment, as summarised in Fig. 9. These data illustrate that the Acetaminophen ‘toxic’ exposure induced significant caspase activity, with no increased activity for Azathioprine ‘toxic’ exposure as measured on day 7.

Caspase activity was also observed for the positive control tamoxifen, while the therapeutic exposures for both Acetaminophen and Azathioprine treatment showed no detectable caspase activity on day 7 of the dosing scheme treatment.

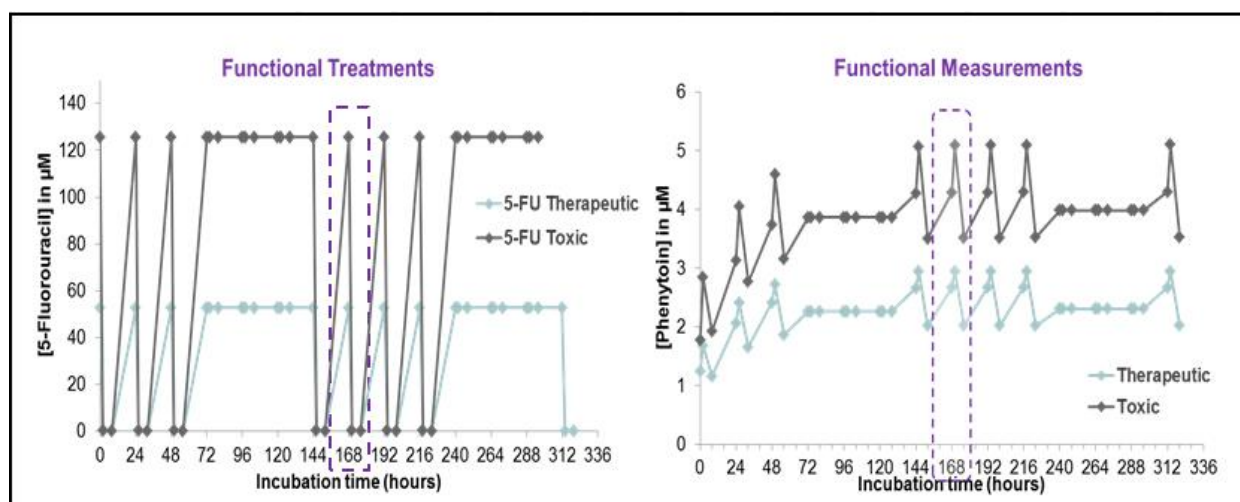


**Fig 8:** Graphical representation of 7 day long-term drug treatments of Acetaminophen and Azathioprine.

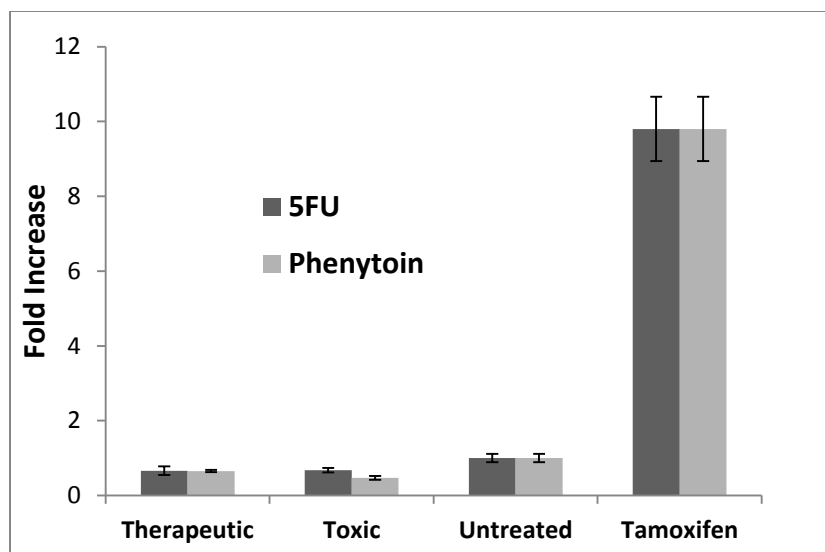


**Fig 9:** Caspase activity resulting from Acetaminophen and Azathioprine treatment measured 7 days post initial treatment, using the complex dosing schemes for both 'toxic' and 'therapeutic' conditions.

Complex dosing schemes delivered from work package 4 for 5-Fluororacile and Pheytoin (Fig. 10) were applied to liver spheroids, where the ability of the compounds to activate caspase activity in liver spheroids was assessed 7 days post initial treatment, as summarised in Fig. 11. These data illustrate for both therapeutic and toxic exposures of 5-Fluororacile and Phenytoin treatments showed no detectable caspase activity on day 7 of the dosing scheme treatment.



**Fig. 10:** Graphical representation of 7 day long-term drug treatments of 5-Fluororacile (left) and Phenytoin (right).



**Fig. 11:** Caspase activity resulting from 5-Fluororacile and Phenytoin treatment measured 7 days post initial treatment, using the complex dosing schemes for both 'toxic' and 'therapeutic' conditions.

All data sets have been submitted to Work package 9 data warehousing during this period.

## DIFFICULTIES

It must be noted that the Caspase assay is destructive and so these measurement give just a snap shot of the activity at one given time. Caspase Activity may have occurred earlier during the dosing period and would not be detectable after Day 7, because the dosing schemes used involve regular media removal and re-dosing which complicates caspase measurements as any released enzyme is removed. Accurate data interpretation requires consideration of these factors.