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Contents

Publishable Summary.....	3
Objectives	3
Introduction	3
Results.....	4
Establishing adrenergic signalling as an important target pathway.....	4
Quantifying toxicity: Linear regression of genes involved in adrenergic signalling.....	7
Clustering of gene expression in adrenergic signalling.....	8
Mechanistic models for adrenergic signalling	10
Simulation of activation/deactivation scenarios with a Hill differential equation model.....	13
Outlook	15
Integrated model of anthracycline cardiotoxicity	15
Difficulties	15
References	16

PUBLISHABLE SUMMARY

In our recent report on mitochondrial signalling (D2.5) we found evidence for adrenergic signalling as a important pathway in the context of cardiac toxicity resulting from anthracyclines. In this report we follow up on these findings. We defer modelling of immune response to a later stage of the project when more data from liver micro tissue will be available. Changes in adrenergic signalling are prominent in our data. Combining our observations with the findings on changes in mitochondrial function as described in the previous report, there is now growing evidence that a combination of oxidative stress and changes in adrenergic signalling may lead to the induction of apoptotic processes. This in turn leads to progressive degeneration of cardiac tissue under the effect of a toxic dose of anthracyclines. Our analysis suggests that this induction may be mediated via the regulation of the BCL-2 gene. BCL-2 is an integral outer mitochondrial membrane protein that regulates cell death by controlling the mitochondrial membrane permeability. Expression changes were much weaker and more difficult to interpret in case of a weaker dose (therapeutic dose). In further analysis we want to use our modelling approach to gain better understanding of competing effects in molecular pathways inducing apoptotic processes.

OBJECTIVES

Aim of the work is to analyze specific molecular aspects of toxicity, in particular aspects mitochondrial dysfunction and immune response. Following the promising results on mitochondrial signalling with evidence for adrenergic signalling as a pathway of interest in the context of cardiac toxicity resulting from anthracyclines the analysis was focused on the adrenergic signalling pathway. The modelling of immune response will be addressed at later stage of the project when the data available is more favourable for models of immune response with more data from liver micro tissue. MPIMG and MD perform computational approaches for in-depth analysis of predictive potential, in particular in specific pathways related to adrenergic signalling which will be used for model refinement in WP3 and WP4 and for the construction of adverse outcome pathways (AOPs) in WP11.

INTRODUCTION

For investigating the effects of anthracyclines on cardiac micro tissue extended molecular datasets were generated in the HeCaToS project. In particular data on idarubicin, doxorubicin, epirubicin are now available, daunorubicin to be added soon. All these drugs are very important cancer therapeutics, albeit with substantial cardiotoxic side effects. These data formed the basis for data analysis and modelling work in WP 2.

By applying the computational pipeline built and described in Deliverable Report D2.3 to the data sets generated in the HeCaToS project, new target pathways for understanding anthracycline mediated toxicity were identified. A pathway prominent in several of our analyses of the molecular data is the adrenergic signalling pathway. Here we report on our efforts for a more detailed analysis of the role of adrenergic signalling in the context of drug induced cardiac toxicity by data analysis and modelling.

The modelling of molecular aspects of mitochondrial dysfunction in respect to cardiotoxic side effects from anthracyclines and the current results showed evidence of an important role of adrenergic signalling. These results were supported from literature where adrenergic signalling is known to be a potential therapeutic target for anthracycline induced cardiomyopathy. There are several studies where beta blockers were evaluated for their protective effect (Georgakopoulos 2010, Kalay 2006, Kaya 2012, Geisberg 2010, Fajardo 2006).

RESULTS

Establishing adrenergic signalling as an important target pathway

Investigating the anthracycline dataset in different aspects we could underpin previous findings pointing to the importance of adrenergic signalling. The first approach reported here draws on the commonality between the different anthracyclines. To be precise we looked for genes that were implied by all three expression data sets investigated here. Differentially expressed genes were identified for the three drugs idarubicin, doxorubicin, and epirubicin and the intersecting gene set was determined as shown in figure 1. An enrichment analysis of this gene set identified adrenergic signalling in cardiomyocytes and different types of cardiomyopathy as common target pathways. Top pathways are listed in the table 1 with their p-values.

Pathway	Adjusted p-value
Dilated cardiomyopathy	2.9E-05
Hypertrophic cardiomyopathy (HCM)	2.5E-05
Cardiac muscle contraction	0.00015
Axon guidance	0.00016
Adrenergic signalling in cardiomyocytes	0.00094
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.00081
p53 signalling pathway	0.00529
Systemic lupus erythematosus	0.00621

Table 1: Top pathways from enrichment analysis of differentially expressed genes for idarubicin, doxorubicin, and epirubicin

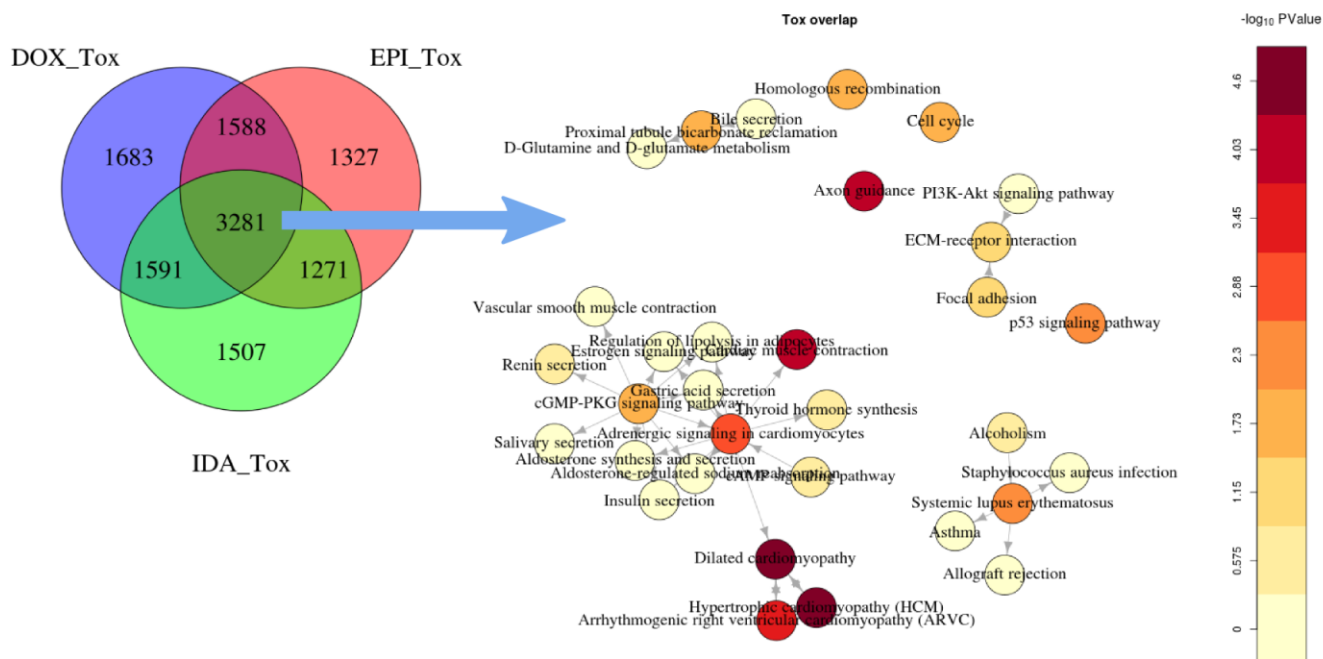


Figure 1: **Overlap of differentially expressed genes for the drugs idarubicin, doxorubicin, and epirubicin in toxic dose** and an enrichment analysis of the common DEGs. Changes in cardiac muscle contraction and cardiomyopathy are at the top of the list followed by adrenergic signalling.

In a second analysis we tried to analyse the temporal structure in our data. Progression of molecular events is monitored at seven time points. Of obvious interest are molecular switching events at or close to these time points. In order to identify genes with expression switches at early, midterm and late stages we defined idealized time courses with a switching behaviour at these time points. Proper genes are then identified by correlation analysis, i.e. searching for genes showing a similar time course as our idealized template. In figure 2 we give an example showing genes with an expression switch in the medium time range (after 72h) for cardiac micro-tissue treated with epirubicin. The set is separated for up and down-regulated genes. Among the genes with a midterm expression switch there are three transcription factors (MYCN, GATA5, NFIX), several myosin genes (MYL3, MYL4, MYL9, MYH7) and a DNA methyltransferase (DNMT1) that appeared in previous analysis as possible source for dysregulation of methylation in our drug-treated microtissues. In order to identify molecular function associated with this set of switching genes we performed a pathway enrichment analysis. Again adrenergic signalling is among the most significant pathways showing up (Figure 3). Having established adrenergic signalling as an important target in our data we proceeded to take a closer look at genes and mechanisms being part of the pathway.

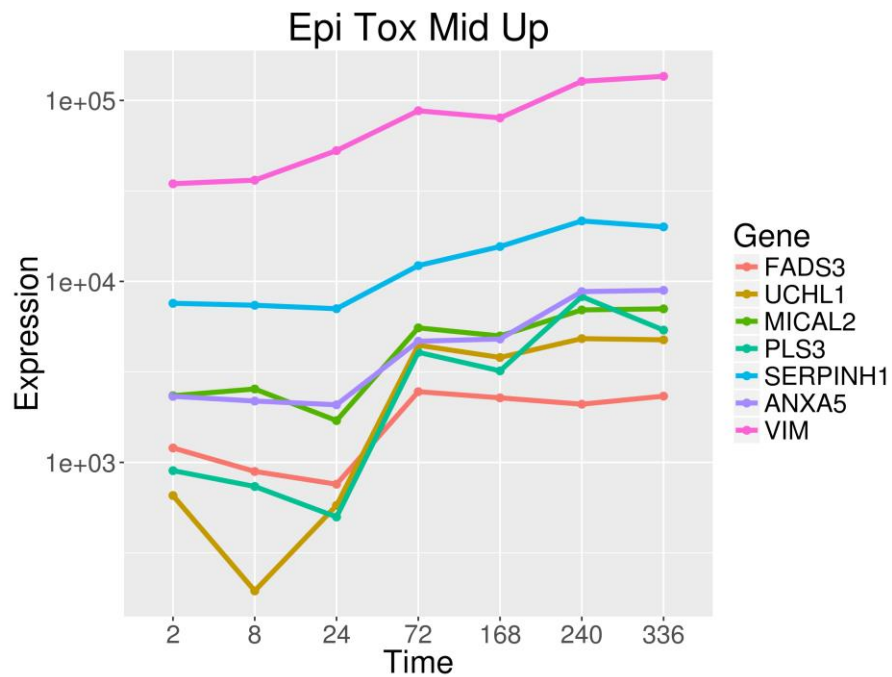
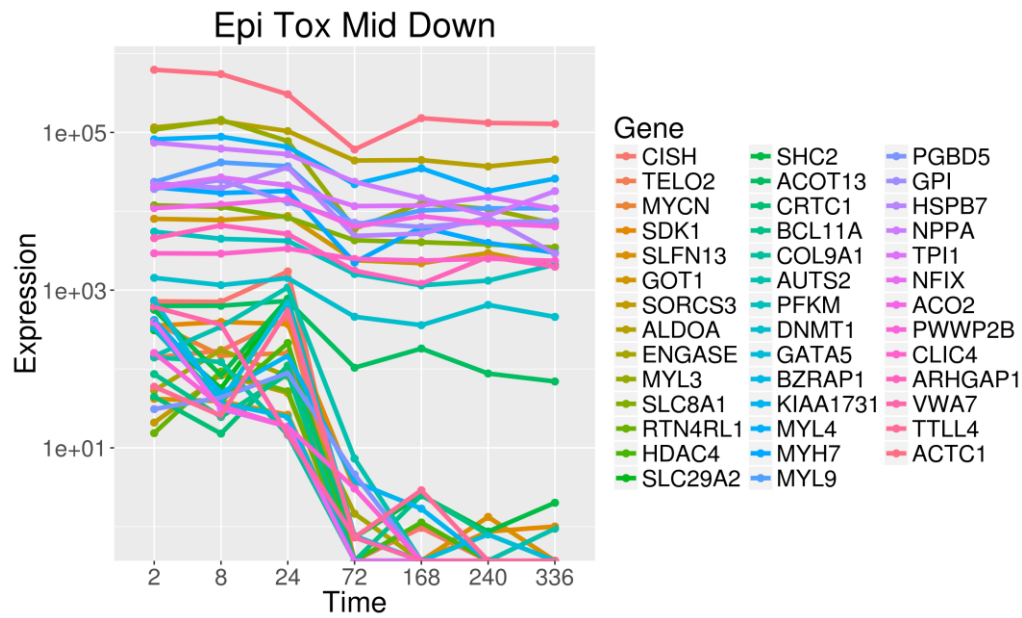


Figure 2: Gene expression with a switch in the medium time range (after 72h) for cardiac micro-tissue treated with epirubicin. Above: down-regulated genes Below: up-regulated genes.

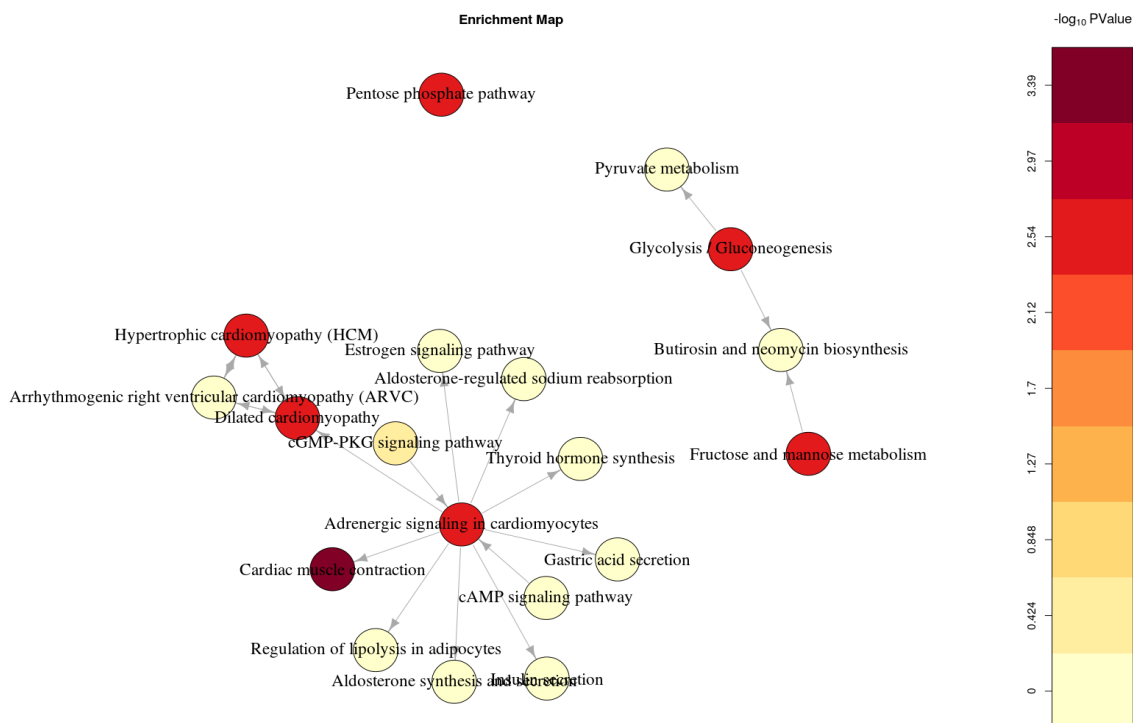


Figure 3: Pathway enrichment map of genes with an expression switch at 72h. Besides cardiac muscle contraction, cardiomyopathy and adrenergic signalling, several metabolic pathways are changed.

Quantifying toxicity: Linear regression of genes involved in adrenergic signalling

The HeCaToS dataset gives us the option to investigate toxic effects of anthracyclines in a quantitative manner with a specific focus on genes in the adrenergic pathway. Quantification is achieved in terms of steepness of response (slope) by fitting linear models to gene expression data. In figure 4 we show the distribution of the slopes for all genes in the adrenergic signalling pathway. The slope is measured in units of [logfold-change per hour]. Thus a value of -0.01 corresponds to a change of $\exp[0.01 \times 140] \sim 4.0$ after five days.

Doxorubicin and epirubicin show a graded response from DMSO to therapeutic to toxic dose, most of the genes being down-regulated. With idarubicin the transition appears to be more non-linear in the sense that no shift is observed from DMSO to therapeutic dose, but than a substantial shift under the toxic dose.

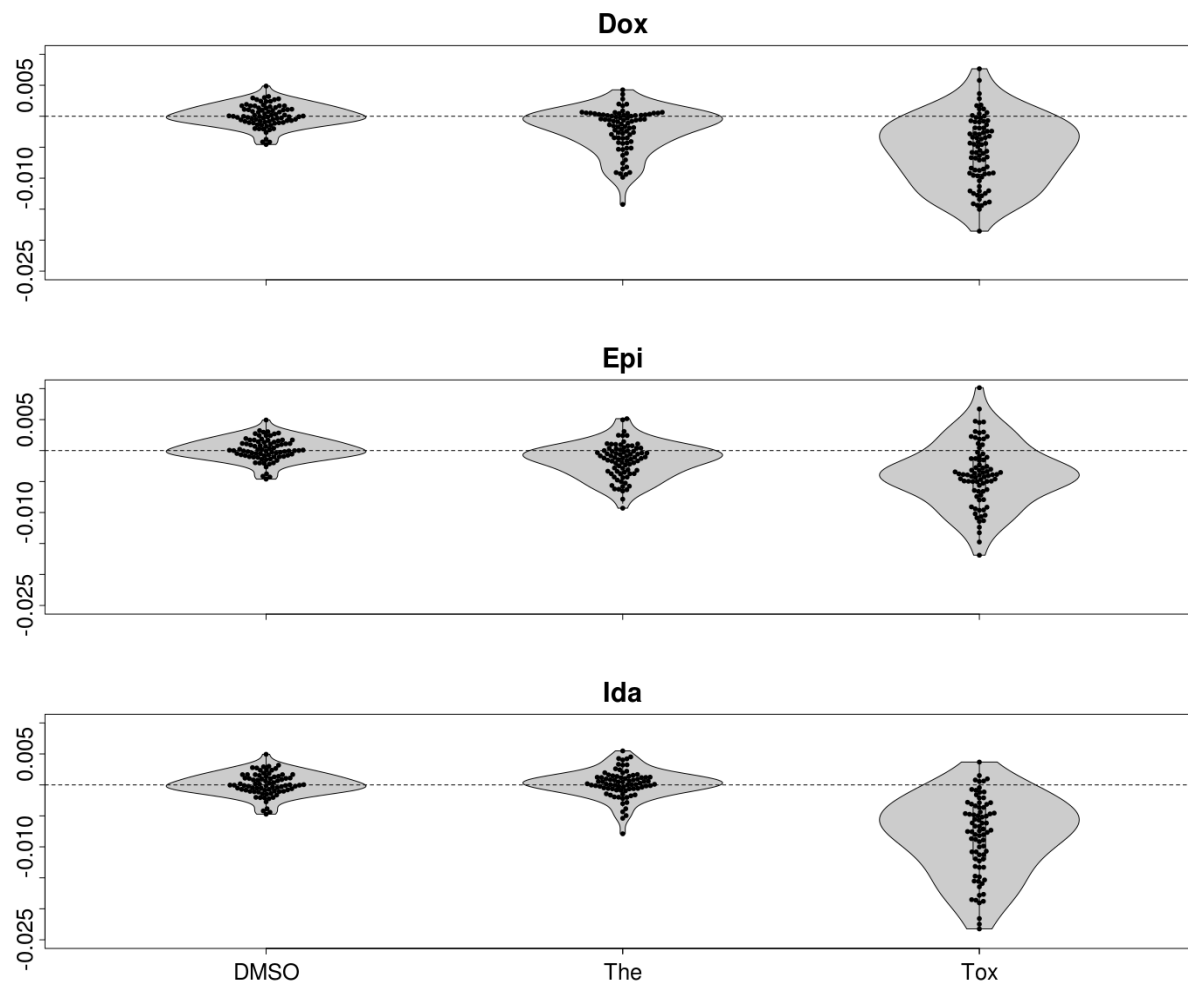


Figure 4: Violin plot giving the slope of linear regression of genes involved in adrenergic signalling. Doxorubicin and epirubicin show a graded dose response while the effect in idarubicine appears to be more non-linear.

Clustering of gene expression in adrenergic signalling

By clustering all genes present in the adrenergic signalling pathway based on their log₂ fold change as compared to fluctuating DMSO we identified groups of genes with similar expression patterns. We used euclidean distance and the tree was cut at an optimal level. This lead to 23 clusters, 6 of them are shown exemplarily in figure 6. In the first cluster all genes show a log fold change increase over time. The other clusters show different types of decreasing expression patterns. In cluster 2 we see a mostly monotonous decrease followed by a slight increase in the later time points. Cluster 3 shows a switching behaviour: it has a steep initial decrease that is compensated at midterm stages followed by a second decrease and a small final increase. Both genes are key components in inhibition and activation of apoptosis. In cluster 4 we observe a fold change decrease until 240h and a steep increase to the last time point that compensates for most of the decrease. Genes of cluster 5 show a basically stable log fold change until 240h and a steep decrease to the last time point. Cluster 6 exhibits an initial decrease followed by a slight increase from 24h to 72h and a steeper decrease in later time stages.

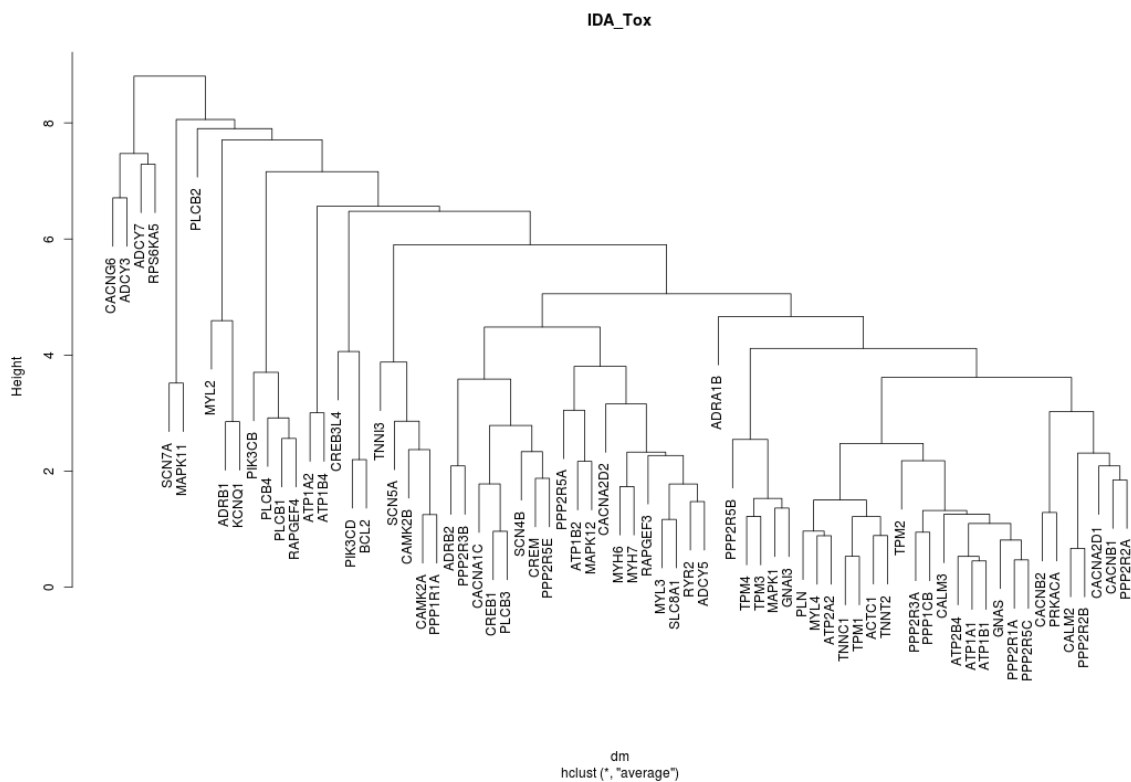


Figure 5: Hierarchical clustering of genes involved in adrenergic signalling

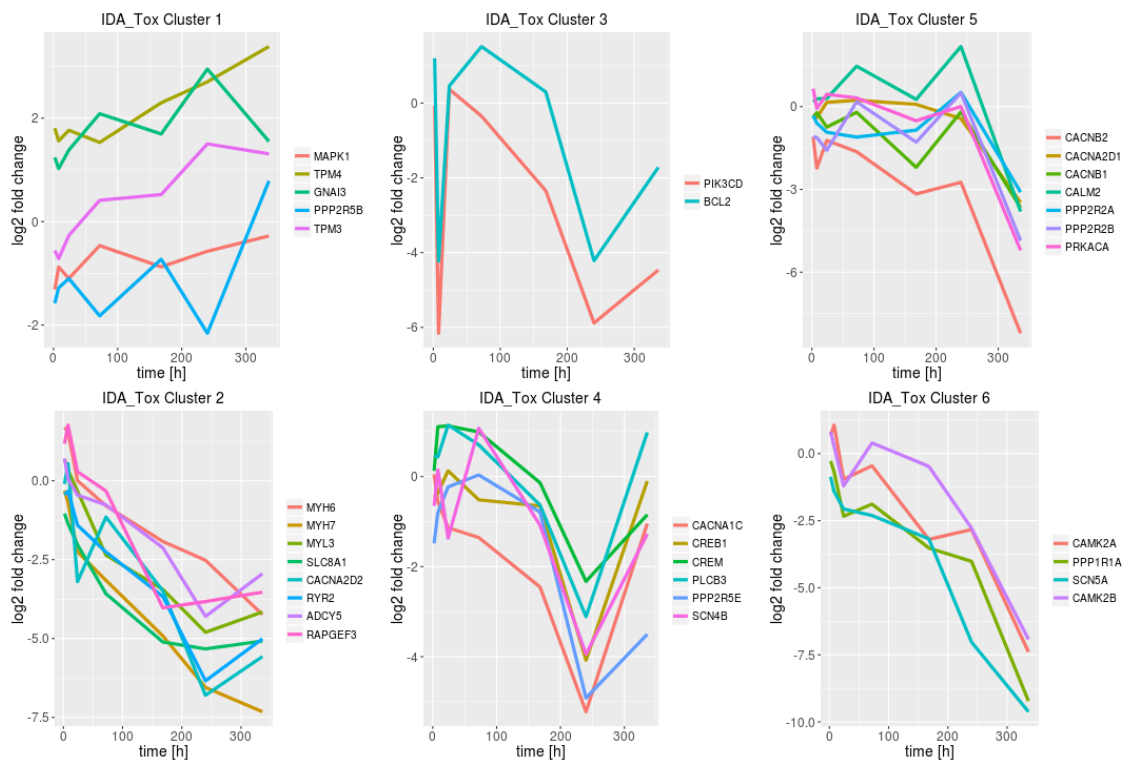


Figure 6: Six selected clusters of genes involved in adrenergic signalling

Mechanistic models for adrenergic signalling

We performed a literature search to identify established models of the adrenergic signalling pathway. Different modelling approaches could be found. Of recent interest are particular the work of Saucerman & al. (2003), Kraeutler & al. (2010), and Shin & al. (2014). A focus of the Saucerman (2003) lies in extending adrenergic signalling pathways with excitation-contraction coupling in rat cardiac cells. The work of Kraeutler (2010) is of methodical interest, combining normalized Hill differential equations with logical operators that allows quantitative predictions even in systems with limited biochemical data. Of particular interest to our investigation is the work of Shin & al. (2014). In this work a connection of adrenergic signalling to the apoptotic switching hub BCL-2 is established.

Figure 7 shows the key feedback loops in the model of Shin et al. The authors identified a switch in BCL2 expression induced by increasing levels of isoproterenol that determines the cell fate of cardiomyocytes.

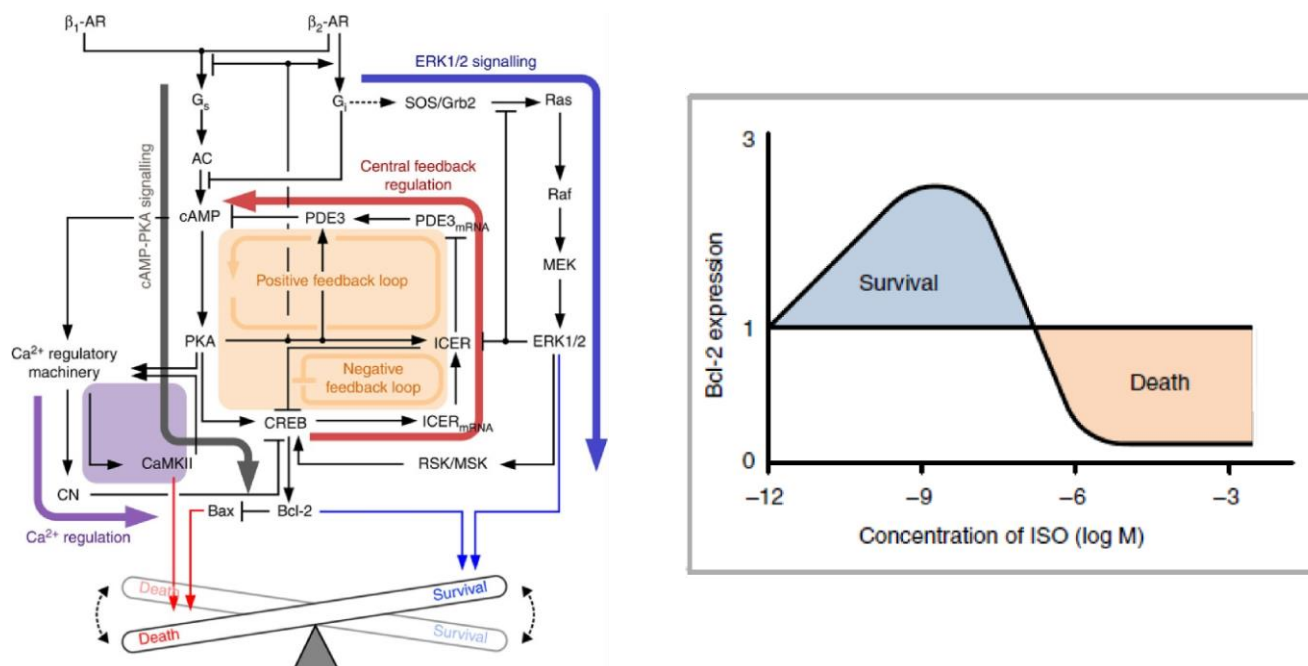


Figure 7: Key components of model of adrenergic signalling from Shin et al. The authors analyse BCL expression as key indicator for cell survival or death.

We identified key genes in these models and associated these genes with available mRNA expression levels. Figure 8 shows line plots of log fold-changes to fluctuating DMSO of three key genes. BCL2 is down-regulated in toxic doses of doxorubicin, epirubicin and idarubicin. Down-regulation can be observed as well in therapeutic dose of doxorubicin. It shows a non monotonous decrease in expression slightly resembling the expression switch as shown in figure 7. BCL2 is known to regulate cell death by controlling the mitochondrial membrane permeability. It appears to function in a feedback loop system with caspases and inhibits caspase activity either by preventing the release of cytochrome c from the mitochondria and/or by binding to the apoptosis-activating factor APAF1 (Marsden 2002). Thus, BCL2 and its interacting partners provide a link from adrenergic signalling to mitochondria.

Phospholamban (PLN) is a key regulator of cardiac diastolic function and a major substrate for the cAMP-dependent protein kinase in cardiac muscle. Unphosphorylated it is an inhibitor of cardiac muscle sarcoplasmic reticulum Ca(2+)-ATPase. In our data, PLN expression is stable for therapeutic doses but decreased in toxic doses. PDE3B is a cyclic nucleotide phosphodiesterase with a dual-specificity for the second messengers cAMP and cGMP, which are key regulators of many important physiological

processes. Through binding to RAPGEF3 and PIK3R6 it assembles a signalling complex in which the PI3K gamma complex is activated by RAPGEF3. The HeCaToS RNA-seq data show a relatively stable PDE3B expression in Epirubicin and Idarubicin therapeutic dose and significant expression changes in all toxic doses and Doxorubicin therapeutic.

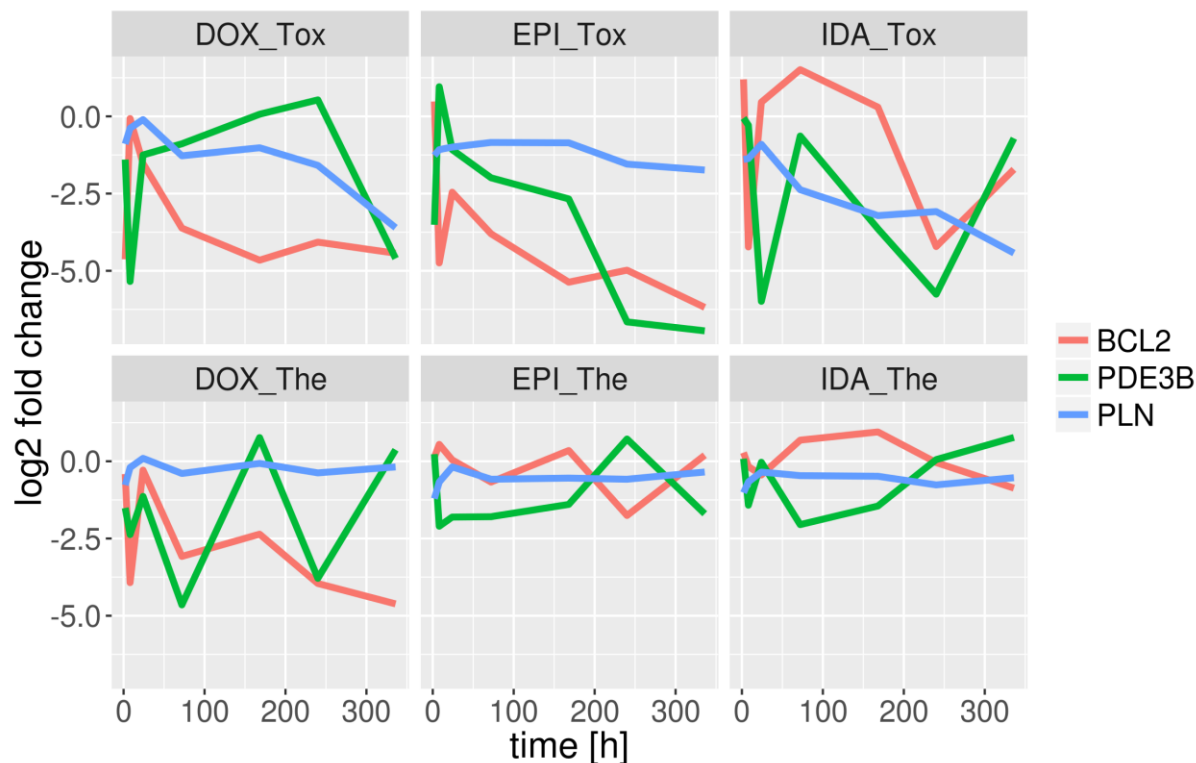


Figure 8: Line plots of fold changes to fluctuating DMSO for key players in adrenergic signalling. Above: action of toxic doses of different anthracyclines on cardiac micro-tissue. Below: action of therapeutic doses.

Motivated by different observations in our data and based on information in pathway databases and in the literature we proceed to formulate a molecular network of BCL-2 regulation. BCL-2 protein is a central survival factor and is under effect of several converging pathways (figure 9). Since regulation acts in part on gene expression in some instances mRNA species are explicitly incorporated. This helps to make dependencies more transparent. The main components in our model are:

1. BAD mediated inactivation of BCL-2. BAD is regulated via PI3K and AKT/PKB,
2. CREB, which is a positive regulator of BCL2 gene on transcription level. PKA and AKT/PKB both result in activation of CREB,
3. PUMA protein inhibiting BCL-2 by binding to its BH3 domain. PUMA transcription is activated by p53u

CREB mediated activation of BCL transcription is partially inhibited by a negative feed-forward loop involving ICER. ICER itself is regulated by the MAP kinase pathway which is not incorporated in this model.

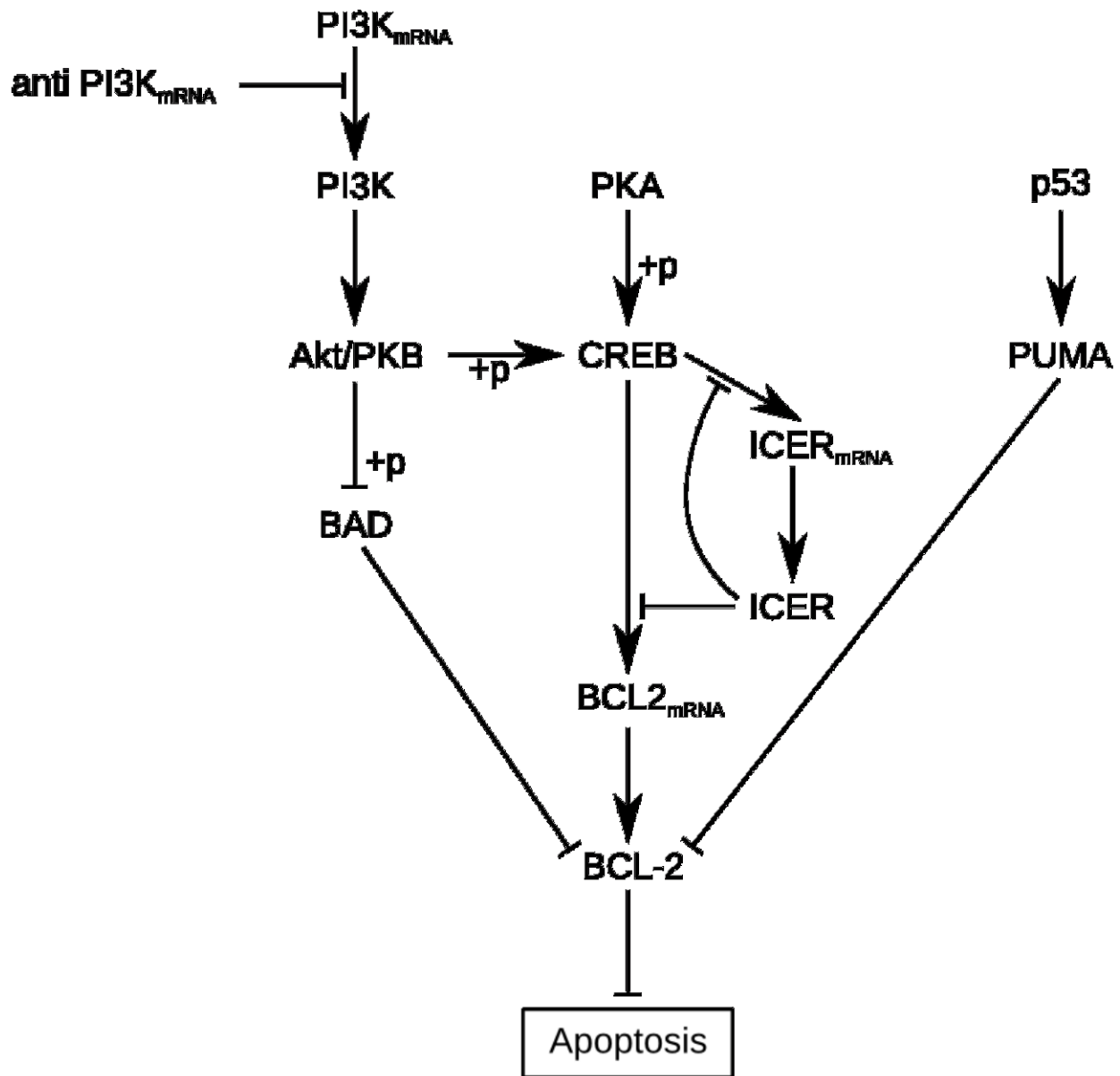


Figure 9: Converging pathways of cell survival. Several pathways are converging on BCL-2, a major hub in regulation of apoptosis. Mutual interaction between different branches of this network makes it an interesting candidate for quantitative and qualitative modelling. ICER, BCL-2 and PI3K are controlled on gene and protein level. In order to make this more transparent the corresponding mRNA species are incorporated in the model. For PI3K we observe changes in anti-sense mRNA which is represented in the model.

Figure 10 shows time dependent mRNA levels of several players involved in the different pathways of our network. PIK3K antisense is strongly down-regulated. This can lead to a positive (pro survival) effect on BCL via deactivation of BAD. The signal of CREB shows a complicated behaviour in time and is not identical in the different conditions. The most dramatic effect on apoptosis probably comes from up-regulation of BBC3 encoding the PUMA protein. This gene is strongly up-regulated in toxic condition (factor 10-15) and less but still clearly up-regulated in therapeutic condition (factor 4). According to current understanding this should result in suppression of BCL-2 and induction of apoptosis. Although some of the observations seem to suggest survival signalling, the finding of strong and early regulation of BBC3 gives a possible explanation of a dominating apoptotic effect mediated on the protein level.

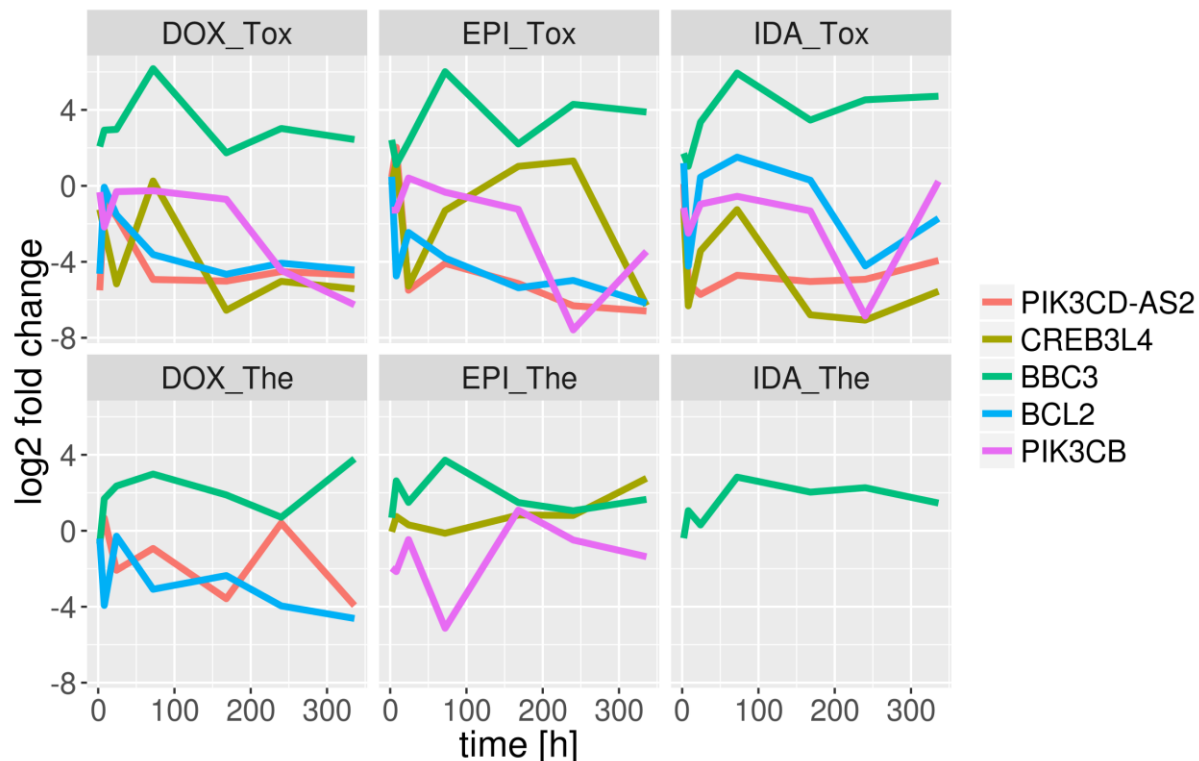


Figure 10: Time dependent activity of several players involved in different pathways of BCL regulation. Of particular interest is the rapid and strong up-regulation of BBC3 which is the gene encoding PUMA protein. PUMA is known as a potent inhibitor of BCL-2 protein and thus inductor of apoptosis.

Simulation of activation/deactivation scenarios with a Hill differential equation model

We use the logic-based Hill differential equation model to gain some insight into some typical regulation scenarios for BCL-2 (Kraeutler et al. 2010). Species dynamics are predicted using ordinary differential equations, where the state variables represent fractional activation of each species. Species interactions were defined with normalized activating or inhibiting Hill functions (f_{act} or f_{inhib}) which are described below. Pathway crosstalk is implemented using logical AND and OR operations. In our simulations presented below these values are constrained to unity. The normalized activating or inhibiting Hill functions have the following form:

$$f_{act}(X) = BX^n / (K^n + X^n); f_{inhib}(X) = 1 - BX^n / (K^n + X^n)$$

where B and K are constrained such that $f_{act}(0) = 0$, $f_{act}(EC_{50}) = 0.5$ and $f_{act}(1) = 1$. From these constraints, it can be derived that:

$$B = EC_{50}^{n-1} / 2EC_{50}^{n-1}; K = (B - 1)^{1/n}$$

We further constrained $f_{act}(X) = 1$ for $X \geq 1$ to ensure that species activities are limited to Y_{MAX} . As default parameters, we used $W = 1$, $EC_{50} = 0.5$, $n = 1.7$, $\tau = 1$, and $Y_{MAX} = 1$. In this description we closely follow Kraeutler et al. 2010 more details can be found in the original publication.

The first scenario is described by constitutive activation of PI3K-mRNA modulated by a expression pulse of PI3K antisense RNA. Figure 11 shows how a combined effect of BAD suppression and BCL activation (via CREB) leads to up-regulation of BCL activity resulting in an anti-apoptotic effect. The second scenario starts from this survival situation. Now we simulate an activation of P53 leading to an activation of expression of the PUMA protein. PUMA inhibits BCL-2 on protein level. If PUMA is activated sufficiently this pro-apoptotic effect of overwhelm the pro survival signal mediated via CREB leading to apoptosis.

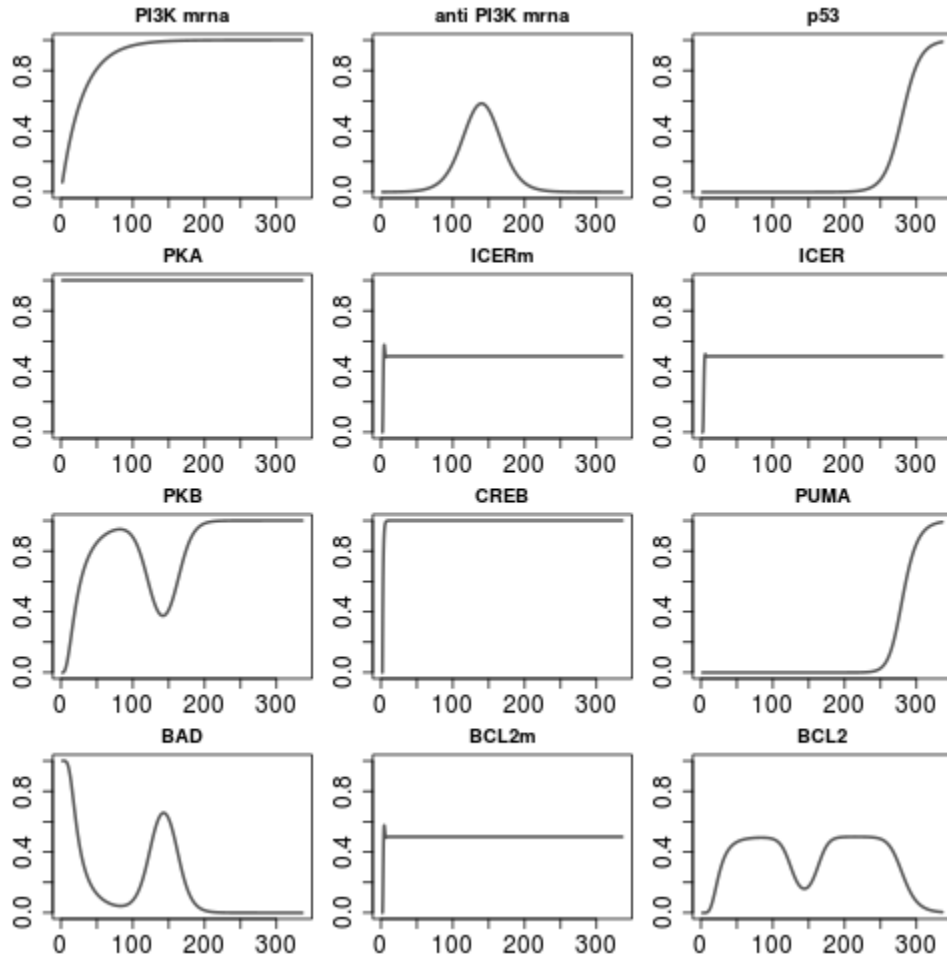


Figure 11: Simulation of different scenarios of BCL2 regulation. A series of time dependent stimuli is applied to the network presented in figure 9. The first stimulus consists in up-regulation of PI3K mRNA (row1, col1) finally resulting in up-regulation of BCL2 (row4, col1) via CREB activation and BAD inhibition. The second stimulus starting around $t=100$ and ending at $t=200$ is a transient up-regulation of antiPI3K mRNA (row1, col2). This stimulus leads to transient activation of BAD (row4, col1) which then leads to transient suppression of BCL2. The last stimulus is

up-regulation of p52 starting at t=250. This stimulus leads to expression of PUMA protein, suppressing BCL-2 activity by protein-protein binding.

OUTLOOK

Integrated model of anthracycline cardiotoxicity

Figure 12 shows possible ways of induction of apoptosis through anthracyclines: it is known that anthracyclines cause oxidative stress and disrupt iron regulation leading to apoptosis through inhibited BCL2 expression. Another source of disturbance of BCL2 expression is a change in adrenergic signalling. Whether anthracyclines directly influence adrenergic signalling or the influence is mediated indirectly through oxidative stress and/or disturbed iron regulation is unclear. Combining all available data, our goal is to extend existing models for adrenergic signalling and combine them with modules for other known causes of anthracycline toxicity.

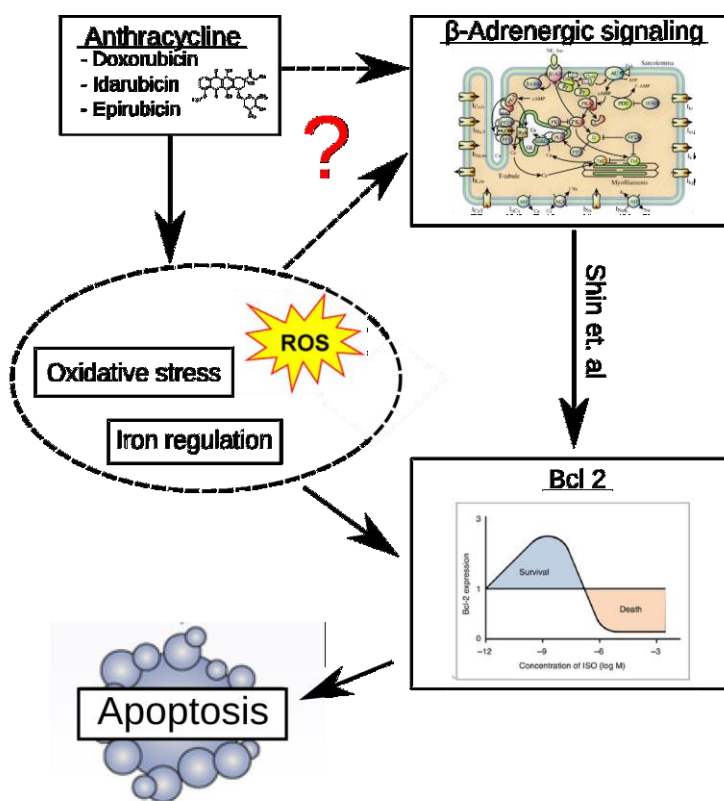


Figure 12: Anthracycline mediated induction of apoptosis. Anthracyclines act in several different ways on the cardiac cell leading to complex changes on the molecular level. Dotted arrows represent effects inferred from our experimental data but still without mechanistic interpretation. We extended the conventional oxidative stress pathway by an alternative route via adrenergic signalling and BCL. BCL2 switching from survival to apoptotic stands here as a representative for several processes mainly located in mitochondrial membrane leading to apoptosis.

DIFFICULTIES

None.

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