# Chapter 5

# Application of Data to Mathematical Models

## 5.1 Introduction

Chapter 4 describes how mathematical models of differing complexity and accuracy were derived in order to describe the kinetics of the cell cycle on the basis of what we can measure in the laboratory. Data with regards to population doublings, senescence and phases of the cell cycle was measured for control cells and those incubated in RHPS4 at concentrations of 50 nM, 100 nM and 1 μM for up to 21 days. The cells were analyzed at days 4, 6, 10, 14 and 21 days.

The data from RHPS4 was applied to the two-, three- and five-compartment models (as derived in chapter 4) to illustrate that the conclusions drawn with regards to RHPS4 are different depending on the mathematical model used as each model has its own assumptions about the cell cycle and uses different aspects of data derived. For example, application of the data to the two-compartment model requires only the doublings data and the cell cycle data is split into two compartments. When applying the data to the five-compartment model, there is the need for data regarding the population doublings, senescence and the proportion of cells in each phase of the cell cycle and therefore utilizing more of the available data. In addition to data regarding the effects of RHPS4 on HCT116 cells, data regarding the effects of doxorubicin was also derived. Doxorubicin has a known mechanism of action and predominantly induces DNA damage in $G_2$ phase cells and there was an increase in the proportion of cells in the $G_2$/M phase[1] (Potter *et* al, 2002). Doxorubicin affects DNA and RNA synthesis, possibly by

[1]HeLa cells treated with 1 μM doxorubicin between 0.5 – 6 hours had greater DNA damage in each cell cycle compartment than in untreated samples, and it was evident that $G_2$/M phase cells were the most susceptible (Potter *et al*, 2002).

reducing RNA chain initiation and through this kills both dividing and non-dividing cells (Barranco, 1975). Due to the different mechanism of action than that already known of RHPS4, doxorubicin will prove useful as a comparative. The data for doxorubicin was gathered in the same way as for RHPS4 with respect to cell type, times and $GI_{50}$ concentration range. The aim of testing the model using data from agents such as doxorubicin with a known mechanism of action was to observe and compare whether the conclusions obtained were the same for both molecules, or if they differed, and if so where did they differ. If, when applied to the model, the conclusions derived for both agents are the same then the model does not offer any information for the individual agents and therefore fails. The five-compartment model, as illustrated in chapter 4, is the most sophisticated of the models that were derived and so the data obtained with respect to doxorubicin was applied to this model only.

Based on the deliberations of chapters 2, 3 and 4, it was decided that the best way forward was to analyse the pre-$G_1$ fraction using the software packages winMDI and Cylchred. The proportions of cells in $G_1/G_0$, S and $G_2$/M will be analysed using the original manual method. In carrying out cell cycle analysis in this way, we are assuming that the proportion of cells in the pre-$G_1$ fraction, and therefore cell death is equivalent to the total DNA intensity of this portion of the histogram divided into the average DNA density of a cell in $G_1/G_0$.

## 5.2 Effect of RHPS4 on HCT116 Cells

### 5.2.1 Effect of RHPS4 on Senescence

Under normal growth conditions, HCT116 cells appear to have a basal level of senescence. As shown in figure 5.1, there is an average of 18.0±2.0% senescent cells present in HCT116 cells under normal conditions. To allow the analysis the effect of RHPS4 on this basal level of senescence in HCT116 cells, they were incubated for up to 21 days with a range of RHPS4 concentrations between 50 nM and 1 μM. At days 4, 6, 10, 14, and 21, the cells were analysed using the SA-β-gal staining method as described in chapter 2. It is evident that RHPS4 induces

senescence in HCT116 cells and the graph in figure 5.1 shows there is both a concentration- and time-dependent effect of RHPS4 on the senescence levels of HCT116. For example, at day 4 under control conditions, there are 18.0±2.9% cells in the senescent phase. Following incubation with RHPS4 at a concentration of 50 nM, the proportion of senescent cells increases to 37.4±2.6% and increases further to 39.2% when incubated in RHPS4 at a concentration of 1 μM. At this early time point, there is no significant difference in the proportions of senescence between the increasing concentrations of RHPS4. Incubation of HCT116 cells with RHPS4 at longer times increases the level of senescence further in a concentration-dependent manner. At day 6, the proportion of senescent cells varies from 35.0±10.3% to 53.0±4.9% between concentrations of RHPS4 at 50 nM and 1 μM, respectively. By day 21, the proportions of senescent cells have increased further to 43.6±2.8% and 84.1% at concentrations of RHPS4 of 50 nM and 1 μM, respectively. This is evidence for RHPS4 having both a time- and concentration-dependent effect of senescence in HCT116 cells as shown in figure 5.1.

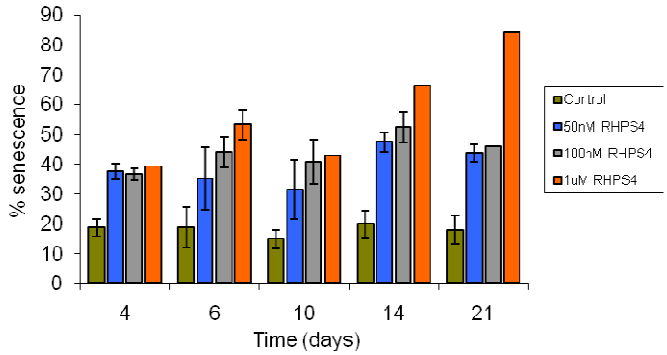



**Figure 5.1 Senescence levels induced in HCT116 cells in response to RHPS4.** The cells were incubated for up to 21 days and analysed at days 4, 6, 10, 14 and 21 for the proportion of senescence using the SA-β-gal staining method.

### 5.2.2 Effect of RHPS4 on Population Doublings

In addition to senescence data, the number of doublings, and therefore overall growth rate of the cells was observed. As with senescence, RHPS4 appears to

have a concentration-dependent effect on HCT116 cells, with higher doses increasing the doubling times as shown in figure 5.2. Under control conditions, there is a consistent doubling time throughout the 21 days, completing an average of 13±0.97 doublings. The addition of RHPS4 reduces this number in a concentration-dependent manner as cells cultured in 50 nM and 100 nM RHPS4 completed an average of 11.5±1.02 and 10.7±0.79 doublings, respectively, in 21 days. Increasing the concentration of RHPS4 to 1 μM reduces the number of doublings to an average 3.54±1.14 in 21 days. Cells cultured in 1 μM RHPS4 appear to reach their maximum number of doublings at approximately 10 days after which there is no net growth of the population of cells as the population stays stable.

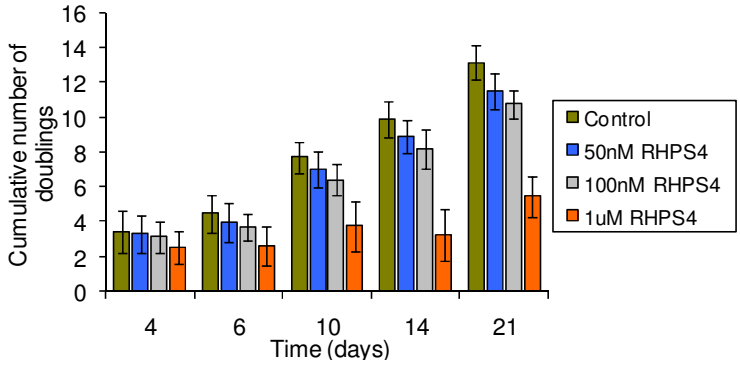



**Figure 5.2 Population doublings of HCT116 cells in response to RHPS4 up to 21 days.** HCT116 cells were incubated in a range of concentrations of RHPS4 for up to 21 days and their population doublings analysed at days 4, 6, 10, 14 and 21. A haemocytometer was used to analyse the population doublings.

### 5.2.3 Cell Cycle Effects of RHPS4

In addition to data concerning the population doublings and senescence levels, data regarding the cell cycle is derived via the Nicoletti method as described in chapter 2. HCT116 cells were stained with PI which penetrates the nucleus of the cell and binds to the nucleotide pair of guanine and cytosine with little or no sequence specificity (Suzuki *et al*, 1997). The cells are then passed through a flow cytometer which analyses the DNA content based on PI fluorescence allowing us to establish what stage(s) of the cell cycle the cells are in at that specific time.

Analysis of cells incubated under control conditions reveals that in addition to having a basal level of senescence, there are also a proportion of cells in pre-$G_1$, which are simply cell degradation products or dead cells. Throughout the 21 days of the experiment, it appears that control cells had approximately 5.3±1.3% cells in pre-$G_1$, whilst there was 53.8±4.8% residing in the $G_1/G_0$ phase of the cell cycle. Equal proportions appeared to be in both S and $G_2$/M phase, those being 21.1±1.3% and 18.4±3.4%, respectively. This cell cycle distribution was maintained in the control cells throughout the 21 days of the experiments.

The addition of RHPS4 to the culture medium perturbs the cell cycle, even at the lowest concentration of 50 nM, which appeared to have no significant effect on the number of doublings. Whilst having no effect on the proportion of cells in pre-$G_1$, there is a reduction in the proportion of cells in both the S and $G_2$/M phases of the cell cycle. The most significant change is observed in the proportion of cells in $G_1/G_0$, which increases by approximately 10% compared to control cells to 63.7±3.4%. As with control cells, this cell cycle distribution is maintained throughout the period of the experiment, with no further significant increases in the proportion of cells in $G_1/G_0$ or changes in pre-$G_1$, S or $G_2$/M. As the concentration of RHPS4 increases, this trend persists: increasing the concentration of RHPS4 to 100 nM has no effect on the proportion of pre-$G_1$ cells, but again reduces the proportion of cells in the S and $G_2$/M phases whilst increasing the proportion of cells in $G_1/G_0$ (approximately 13% above that of the control to 66.7±6.4%) in a concentration-dependent manner. In the presence of RHPS4 at a concentration of 1 μM (i.e. the $GI_{50}$ concentration), there is a further increase in the proportion of cells in the $G_1/G_0$ phase (approximately 14% above that of the control to 67.1±7.4%). Again there is a reduction in the proportion of cells in both the S and $G_2$/M phases of the cell cycle in comparison to the control cells and the lower concentrations of RHPS4 (i.e. 50 nM and 100 nM) used to 14.2±4.2% and 12.6±2.7%, respectively. There appears to be no effect on the proportion of cells in pre-$G_1$.

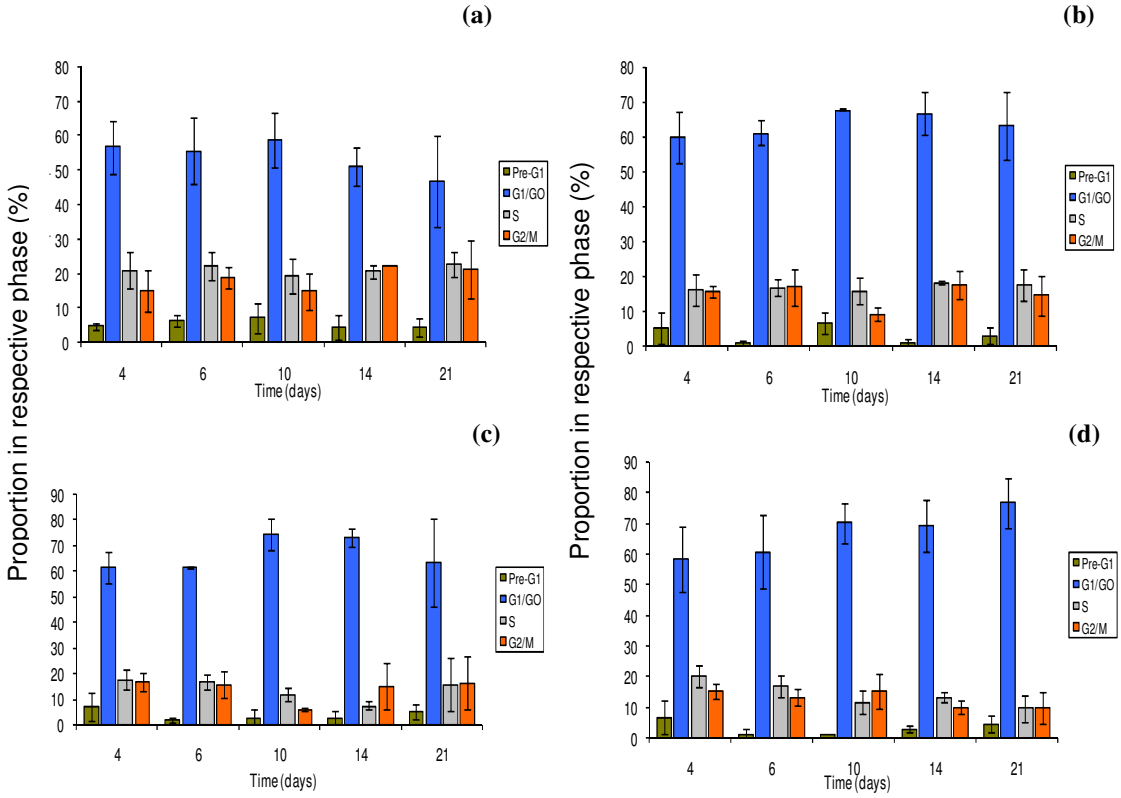



**Figure 5.3 Cell cycle responses of HCT116 cells to RHPS4 up to 21 days.** The cells were incubated with RHPS4 at concentrations of (b) 50 nM, (c) 100 nM and (d) 1 µM. The cells were also incubated in (a) RPMI tissue culture media and their cell cycle analysed at days 4, 6, 10, 14 and 21 days using the flow cytometer where 20 000 events were measured.

### 5.2.4 Summary of the Effects of RHPS4 on HCT116 Cells

In general, it appears that RHPS4 not only has a time-dependent effect, but also a concentration-dependent effect on the cell cycle distribution of HCT116 cells. It appears that RHPS4 causes cells to accumulate in the $G_1/G_0$ phases of the cell cycle and thereby reducing the proportion of cells available for cycling and so we observe a reduction in the proportion of cells in the S and $G_2$/M phase of the cell cycle.

Under control conditions the cells maintain a cell cycle distribution of approximately 5% in pre-$G_1$, approximately 50% in $G_1/G_0$ and approximately 20% in both S and $G_2$/M. This cell cycle distribution was maintained throughout the period of the experiment. The addition of RHPS4 perturbs this distribution,

therefore suggesting that its mechanism of action, in addition to telomerase inhibition, also has cell cycle mediated effects.

At a concentration of 50 nM RHPS4, there is no effect on the proportion of dead cells, however, there is an increase in the proportion of cells in $G_1/G_0$ in comparison to control cells by day 4 of the experiment and this increase is maintained in a time-dependent manner until day 21. There is also a reduction in the proportion of cells in S and $G_2$/M phases in comparison to control, and again this is achieved by day 4 and maintained until day 21 without any significant changes.

At the $GI_{50}$ concentration of 1 μM, the effects of RHPS4 on HCT116 cells were more pronounced, but were slightly longer in becoming established. As with the lower doses of RHPS4, there was no effect on the proportion of pre-$G_1$ cells which remained stable throughout the period of the experiment. At day 4, there was no effect on the proportion of cells in $G_1/G_0$, however, by day 10 the proportion of cells in this phase had risen to approximately 70.1±6.4% and was maintained for the rest of the experiment. The proportion of cells in S phase did not significantly change in comparison to control; however, the proportion of cells in $G_2$/M was reduced by day 4 to 13.1±2.8% and even further reduced to 9.7% which remained for the duration of the experiment.

## 5.3 Application of Data to the Two- and Three-Compartment Mathematical Model

The data was applied to the two-, three- and five- compartment model and the change in the individual rate parameters in comparison to the control cells was analyzed. It is possible to show the changes in the individual rate parameters with respect to both time and concentration, however, any changes observed may not be actual changes due to the presence of RHPS4 as changes also occur in control cells. For this reason, the graphs shown in figure 5.4 - 5.6 indicate changes of each of the rate parameters with respect to time for each of the individual concentrations of RHPS4 used. The graphs in figure 5.4 - 5.6 show changes in

comparison to control and therefore any line above the X-axis is indicative of an increase above control cells, whereas a line below the X-axis is indicative of a reduction. Comparison to control was calculated as $\frac{k_{agent}-k_{control}}{k_{control}}$. As the data from RHPS4 suggests, there is a basal level of senescence and cell death at control conditions and so the two- and three-compartment model would not be expected to be accurate. Even though this is the case, the data was applied to both the two- and three-compartment model to illustrate that depending on which model is used, application of the same data will derive different conclusions regarding the effects of RHPS4 on HCT116 cells.

The data in sections 5.2.1 – 5.2.4 was applied to the mathematical models as shown in section 4.7 to yield values for the corresponding 'k' values for the two- and three- compartment model in comparison to control are shown in figures 5.4 – 5.5, respectively. The data derived from the application of the data to the two- and three-compartment model does not allow us to derive conclusions that can be explained in a biologically sensible manner as the 'rate constant' do not follow a smooth and logical manner. The two- and three-compartment models are closed, and so all cells are considered to be cycling and figures 5.4 – 5.5 show that some of the rate parameters are consistently above the control values. It is hard to believe that the addition of RHPS4, with anti-tumour activity has the ability to make cells go around the system at a faster rate than control cells. By applying the data to the two- and three-compartment models we are simply highlighting that the same data can be applied to two different mathematical models with their own corresponding ODEs and the conclusions can be very different.

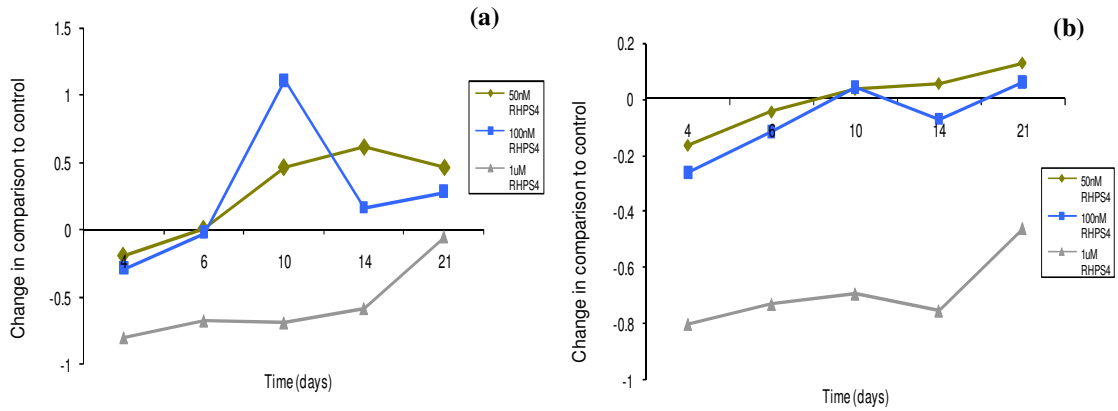



**Figure 5.4 Application of the data from RHPS4 to the two-compartment model.** The data obtained from the analysis of cell cycle and population doublings over 21 days was applied to the two-compartment model to determine rate parameter values for (a) $k_{xy}$ and (b) $k_{yx}$.

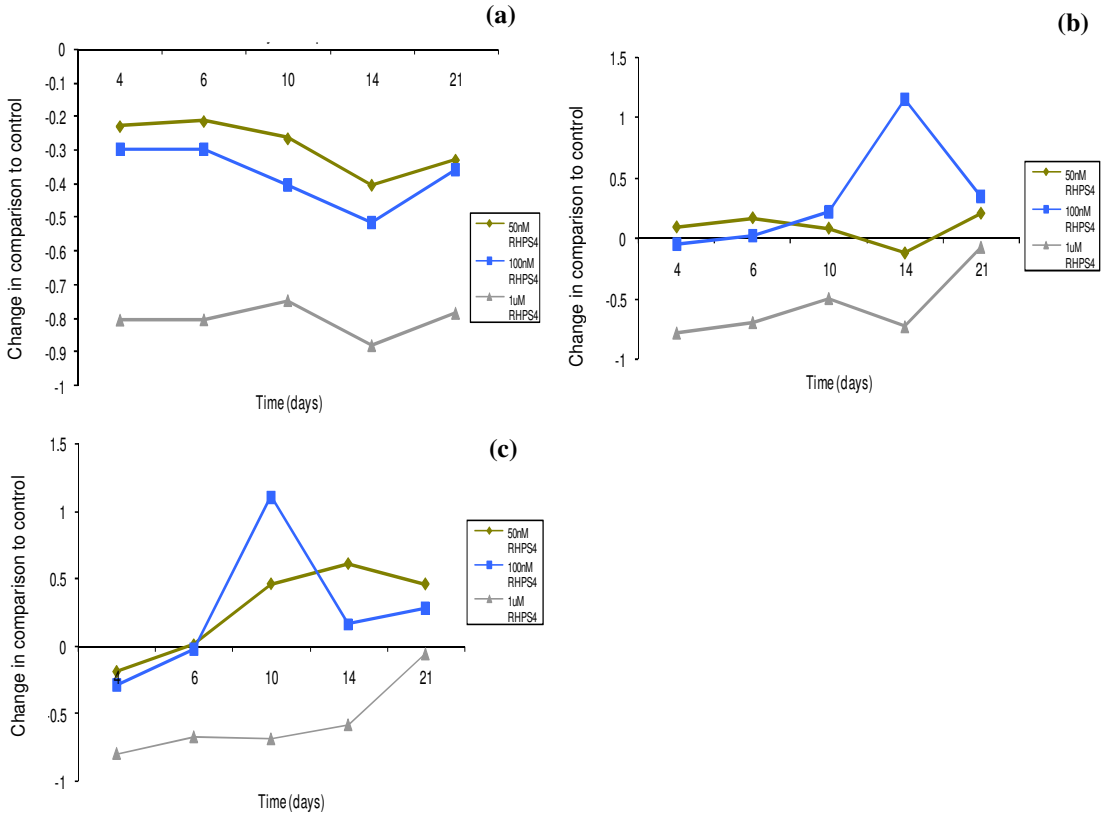



**Figure 5.5 Application of the data from RHPS4 to the three-compartment model.** The data obtained from the analysis of cell cycle and population doublings over 21 days was applied to the three-compartment model to determine rate parameter values for (a) $k_{xy}$, (b) $k_{yz}$ and (c) $k_{zx}$.

## 5.4 Application of Data to the Five-Compartment Model

The data described in sections 5.2.1 – 5.2.3 was also applied to the five-compartment model to determine the effects of RHPS4 on the cell cycle of HCT116 cells and the results are shown in figure 5.6, which shows the effects of RHPS4 at concentrations of 50 nM, 100 nM and 1 μM in comparison to control cells. The data can be analysed independently of the control, however, analysis of the control cells clearly shows that these also change throughout the 21 days of the experiment and so should be taken into account. For this reason, the changes in each of the rate parameters were considered in comparison to the control and so any line above the X-axis is indicative of an increase and any line below is indicative of a decrease below the control. Any line equal to the X-axis would indicate that the value for that rate parameter is equal to the value for control and has therefore not changed.

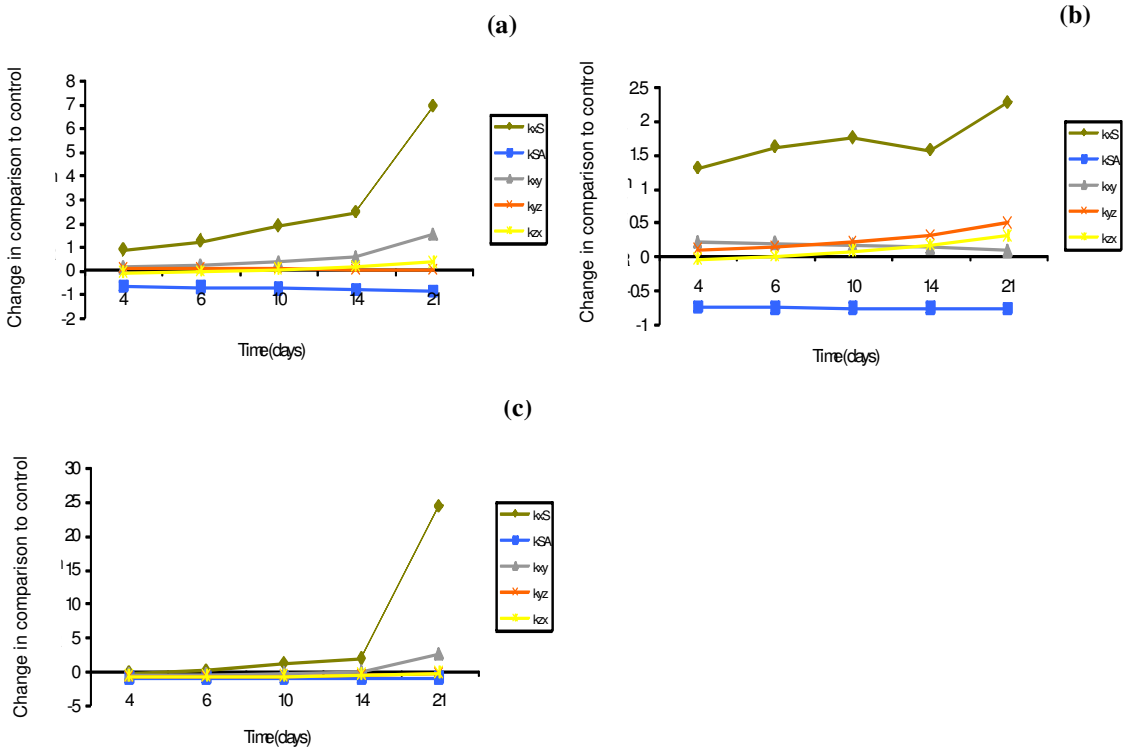



**Figure 5.6 Application of the data from RHPS4 to the five-compartment model.** The data obtained from the analysis of senescence, cell cycle and population doublings over 21 days was applied to the five-compartment model to determine rate parameter values for $k_{xy}$, $k_{yz}$, $k_{zx}$, $k_{x\Sigma}$ and $k_{\Sigma A}$ at concentrations of RHPS4 of (a) 50 nM, (b) 100 nM and (c) 1 µM.

The data applied to the model indicates that at each concentration, there is a time-dependent increase in $k_{x\Sigma}$ and decrease in $k_{\Sigma A}$ from that of the control suggesting there is an increase in the rate at which cells are becoming senescent, however, a reduction in the rate at which they are entering cell death. This reduction in $k_{\Sigma A}$ is less pronounced at the highest concentration of 1 µM, however, still decreases. In addition to the changes in these rate parameters, it is obvious that there is an overall increase in the proportion of cells in the compartments A (cell death) and Σ (senescence). Figure 5.7 shows the proportion of cells in compartments A and Σ individually and as a whole for control cells and in the presence of RHPS4. Figure 5.7b illustrates that in comparison to control, the addition of RHPS4 to the cells at concentrations of 50 nM, 100 nM and 1 µM reduces the proportion of cells in compartment A (indicative of cell death) in a concentration-dependent manner. At the intermediate time points there are anomalies, for example, in the presence of

50 nM RHPS4 there is an increase in cells in compartment A at day 10, however, the concentration-dependent decrease returns by day 14. In addition to this concentration-dependent decrease in the proportion of cells in A, there is both a concentration- and time-dependent increase in the proportion of cells in Σ (indicative of senescence) (figure 5.7a). In control cells the proportion of cells in compartment Σ is stable throughout the 21 day period; however RHPS4 increases the proportion of cells in compartment Σ above the control in both a concentration- and time-dependent manner. Figure 5.7c shows that there is an overall increase in the proportion of cells in compartments Σ and A above the control over the 21 day period due to a decrease in the proportion of cells in compartment A and an increase in the proportion of cells in compartment Σ in a concentration- and time-dependent manner. It is possible therefore to conclude that the rate at which cell leave compartment X to enter compartment Σ via $k_{x\Sigma}$ is increased therefore increasing the proportion of cells here and the rate at which cells leave compartment Σ via $k_{\Sigma A}$ is reduced leading to the conclusion that there is an overall increase in the proportion of cells in compartments Σ and A. Without the mathematical model, the conclusion would indicate that there is this increase in the proportions of cells in these compartments, however, would give no indication as to whether this is due to changes in the kinetics of the cell cycle, and if so, where are the kinetics changing? Application of the data to the model has led to the conclusion that the overall increase in the proportion of cells in compartments Σ and A is due to the overall decrease in the rate parameter $k_{\Sigma A}$ and the increase in the rate parameter $k_{x\Sigma}$. These effects of RHPS4 would be difficult to appreciate without the mathematical model. For example, from the data shown in section 5.2, it is evident that the addition of RHPS4 increases the proportion of senescent cells and reduces the proportion of dead cells, however, does not give any indication as to why this is the case. An increase in the proportion of cells in senescence could be the result of an increase in the rate of cells entering from $G_1/G_0$ or a reduction in the rate of cells entering the cell death compartment. This increase could also be due to some change that has occurred within the cycling population of cells. By

applying the data to the mathematical model we have quantitative data with regards to the effects of RHPS4 on the kinetics of the cell cycle.

Figure 5.6 and the raw data shows that there appears to be no concentration- or time-dependent effect of RHPS4 on the rate parameters $k_{xy}$, $k_{yz}$ and $k_{zx}$ and the rates of these parameters stays relatively close to control values.

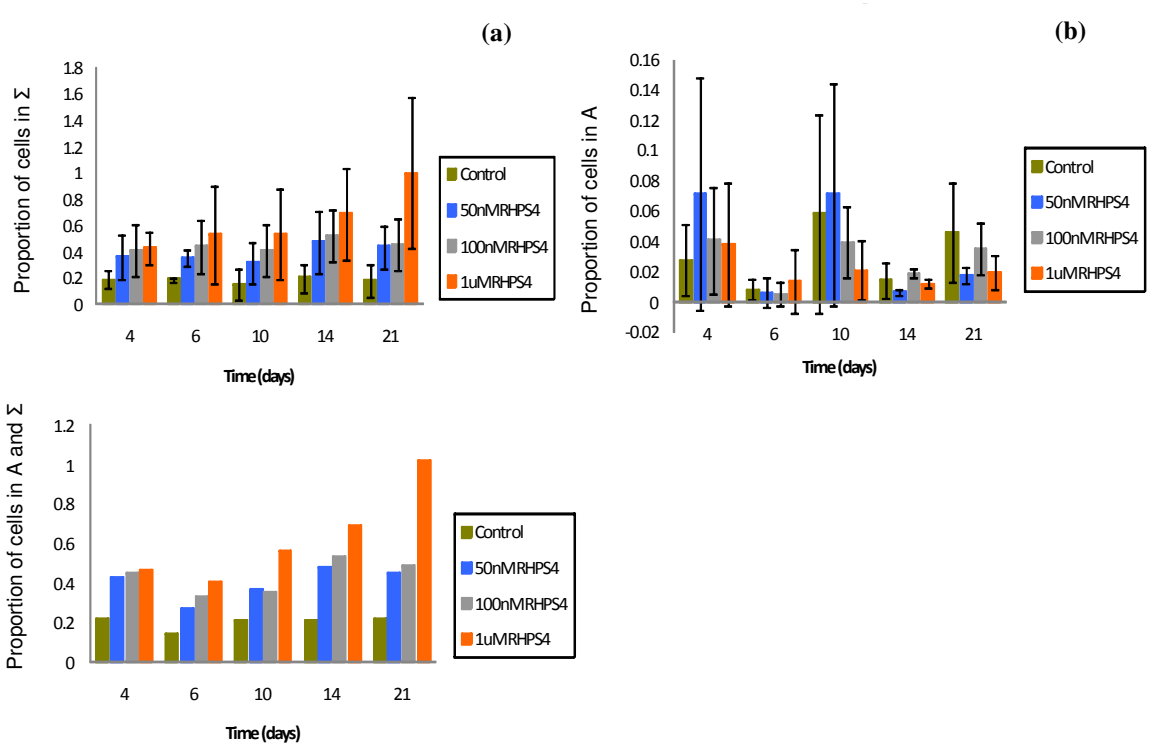



**Figure 5.7 Proportion of cells in A, Σ and A+Σ in response to RHPS4 up to 21 days.** Application of the data obtained from incubation of HCT116 cells with RHPS4 show that there are changes in the rate parameters $k_{x\Sigma}$ and $k_{\Sigma A}$ in comparison to control cells and it is possible that these could be explained by changes in the proportions of cells in (a) Σ, (b) A and (c) Σ + A.

## 5.5 Comparison of the Mathematical Model with Doxorubicin

The same type of data that was generated for RHPS4 was also generated for doxorubicin and applied to the five-compartment model in the same manner as shown in section 5.4. The results of the effects of doxorubicin on the cell cycle, population doublings and senescence are shown in sections 5.5.1 - 5.5.3.

### 5.5.1 Effect of Doxorubicin on the Cell Cycle

The cell cycle of HCT116 cells in response to a range of concentrations of doxorubicin was analysed using the Nicoletti method as discussed in chapter 2. The average proportions of cells in each phase at each time point with respect to concentration are shown in figure 5.8. The proportion of cells in pre-$G_1$ increases with respect to both time and concentration. Under control conditions, it appears that there is a basal level of pre-$G_1$ cells of approximately 5.5±4.9% throughout the experiment. The presence of doxorubicin at increasing concentrations from 3.15 nM to 63 nM increases the proportion of cells in pre-$G_1$. There is also a time-dependent increase in the proportion of cells in pre-$G_1$ in the presence of doxorubicin at increasing concentrations. Incubation with doxorubicin at 63 nM up to day 4, leads to 17.3±12.5% of cells in pre-$G_1$ which increases to 24.8±1.1% by day 21. This time-dependent increase is consistent with each concentration of doxorubicin and is amplified with increasing concentration.

As with RHPS4, doxorubicin appears to have no significant effect on the proportions of cells in $G_1/G_0$. By day 4, control cells have 53.2±12.4% of cells in $G_1/G_0$ and the presence of doxorubicin at a concentration of 3.15 nM changes this 52.4±19.9%. Incubation with doxorubicin at a concentration of 63 nM changes the proportion of cells in $G_1/G_0$ to 53.7±8.8%. These changes are non-significant in comparison to control and it appears that throughout the 21 days, there are no significant changes in the proportion of cells in $G_1/G_0$. Analysis of the proportion of cells in S phase indicates no significant changes in comparison to control cells up to day 4. After day 4, there is a concentration-dependent decrease in the proportion of cells in S phase, however, there appears to be no time-dependent decrease during the experiment. The same pattern is true for the $G_2$/M phase of the cell cycle, however, is present by day 4; however, there is a concentration-dependent decrease in the proportion of cells in $G_2$/M across the 21 days of the experiment.

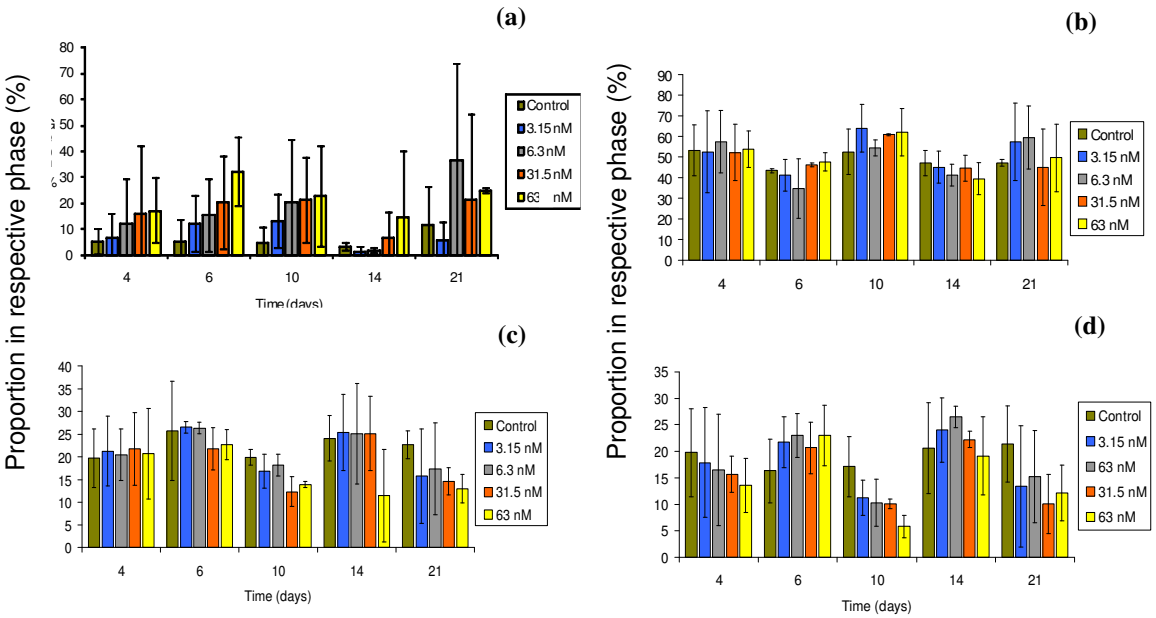



**Figure 5.8 Cell cycle phase changes in response to doxorubicin at a range of concentrations up to 21 days.** HCT116 cells were incubated with doxorubicin for up to 21 days and the cell cycle phases analysed using the Nicoletti method as described in chapter 2. The cells were analysed on the flow cytometer where 20 000 events were measured and the proportions of cells in (a) pre-$G_1$, (b) $G_1/G_0$, (c) S and (d) $G_2$/M were calculated.

As expected, and in parallel with RHPS4, there was a concentration-dependent effect of doxorubicin on the population doublings of HCT116 cells. The population of cells doubled as shown in figure 5.9, at a rate that is not exclusively linear, nor is it exclusively logarithmic. The graph shows that under control conditions, HCT116 cells completed an average 13.4±1.1 doublings over the 21 day period. Incubation with 3.15 nM doxorubicin reduces the number of population doublings to 9.3±0.3 and increasing the concentration further to 6.3 nM reduces the number of population doublings to 7.4±1.9. The number of population doublings is reduced to half of that of control cells in 31.5 nM doxorubicin in which it was determined there were only 4.3±2.9 cumulative doublings over the 21 day period. HCT116 cells incubated with 63 nM doxorubicin, the highest concentration used, significantly reduced the cumulative population doublings to 1.84±1.5 over the 21 days of the experiment. The concentration-dependent reduction in cumulative population doublings is what would be expected of anti-tumour agents, such as doxorubicin and RHPS4.

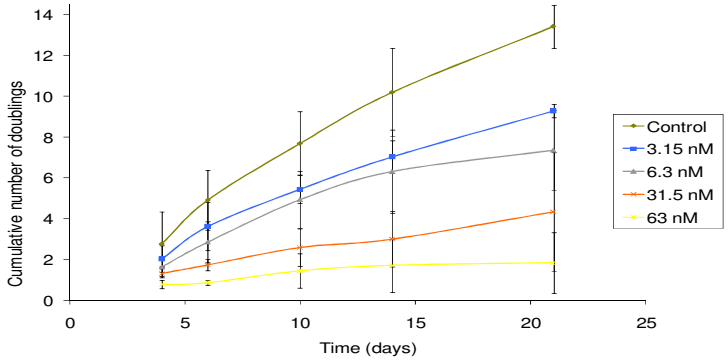



**Figure 5.9 Cumulative population doublings in response to doxorubicin up to 21 days.** The population doublings of HCT116 cells incubated with doxorubicin were analysed using a haemocytometer up to 21 days. The results shown here are the average of n=3.

### 5.5.3 Effect of Doxorubicin on Senescence

The experimental results show both a concentration- and time-dependent effect on senescence in HCT116 cells in response to doxorubicin. The results are shown in figure 5.10 and indicate that control cells display a basal level senescence of approximately 16.3±2.9% over the 21 days of the experiment. Incubation with doxorubicin at a concentration of 3.15 nM increases the proportion of cells in senescence at day 4 to 12.9±5.9% and by day 21, the proportion of cells in senescence increases to 30.9±10.8%. The same pattern is true for each concentration of doxorubicin with the greatest proportion of cells in senescence observed in cells incubated with doxorubicin at a concentration of 63 nM. At this concentration there is an increase from 39.7±18.2% at day 4 to 59.2±8.1% by day 21. The greatest increase in the proportion of cells in senescence throughout the experiment is observed in cells incubated with 31.5 nM doxorubicin. At this concentration there is 17.7±1.8% in senescence at day 4 and increases to 47.3±9.4% by day 21. Incubation of HCT116 cells with doxorubicin at concentrations of 31.5 nM and 63 nM increases the proportion of cells in senescence at each time point to be almost three times that of what is observed in control cells.

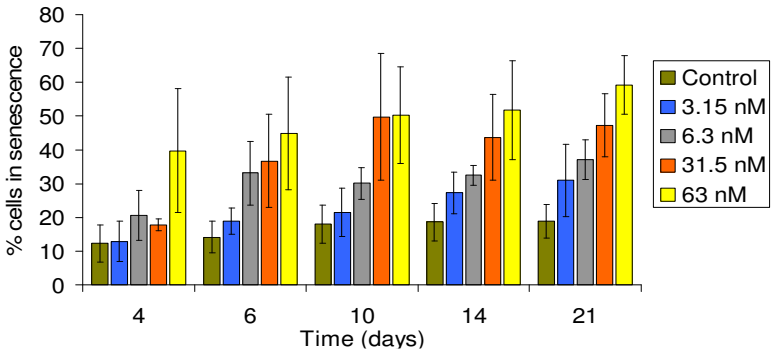



**Figure 5.10 The proportion of cells in senescence in response to doxorubicin up to 21 days.** The cells were incubated with doxorubicin up to 21 days and the senescence levels analysed at days 4, 6, 10, 14 and 21 using the SA-β-gal staining method.

### 5.5.4 Application of the Data Obtained from Doxorubicin to the Five-Compartment Mathematical Model

As previously shown in sections 5.31 – 5.3.3, the different models obtained can allow different conclusions regarding the same data to be derived. The model considered to be the most realistic is the five-compartment model. This model is simple in terms of mathematics and accounts for exit from the system via cell death and senescence. In reality, cell death does not occur only from senescence, however, to keep the model simple it is assumed that the rate of death from the other compartments X, Y and Z is negligible in comparison to that from Σ, and can therefore be ignored. The data described in sections 5.5.1 – 5.5.3 was applied to the five-compartment model in order to compare to the results obtained from RHPS4. If when applied to the model, the conclusions derived are the same for doxorubicin and RHPS4 then the model is not useful as it fails to distinguish between the two molecules. Doxorubicin has a known mechanism of action which involves topoisomerase II inhibition. The mechanism of action of RHPS4, at present, is not yet fully elucidated, however, is known to involve telomerase inhibition possibly due to its high affinity for G-quadruplex DNA. If the model reveals similar conclusions for doxorubicin and RHPS4 then the model requires further refining in order to improve its accuracy.

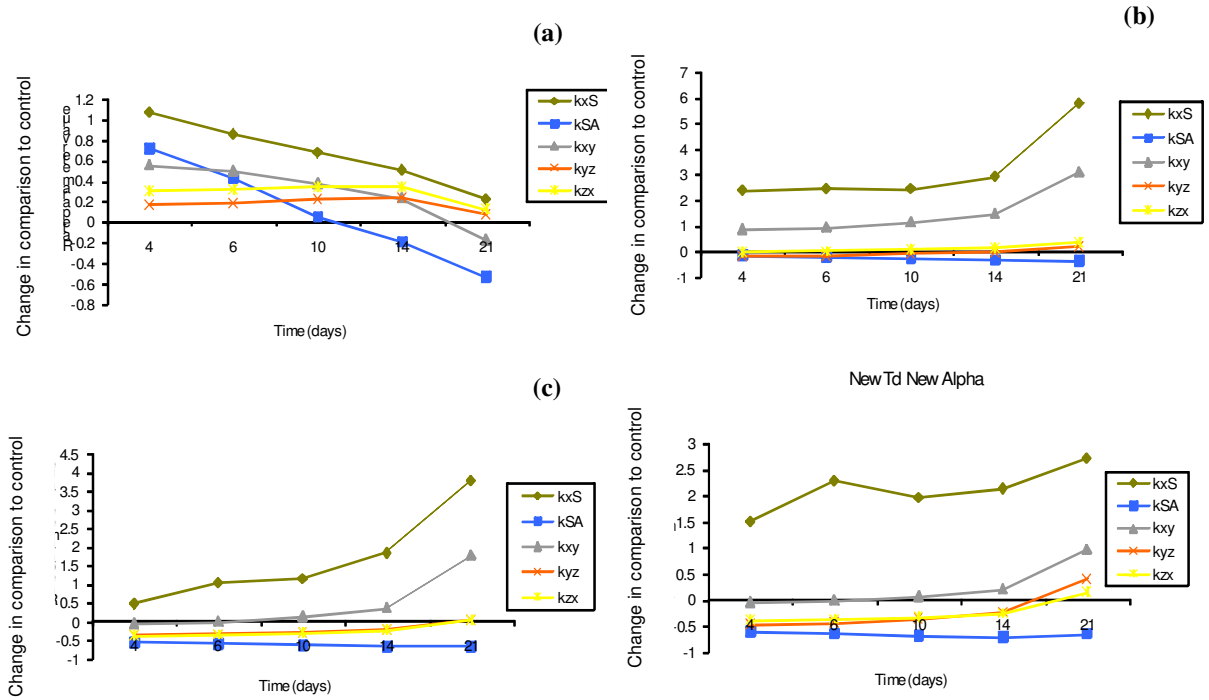



**Figure 5.11 Application of the data obtained from doxorubicin to the five-compartment model.** The data from doxorubicin at concentrations of (a) 3.15 nM, (b) 6.3 nM, (c) 31.5 nM and (d) 63 nM was applied to the five-compartment mathematical model to determine values for the rate parameters $k_{xy}$, $k_{yz}$, $k_{zx}$, $k_{\Sigma A}$ and $k_{x\Sigma}$.

Application of the data shows that in a similar fashion to RHPS4 there is an increase in the rate of cells moving into senescence. The results are shown in figure 5.11 and illustrates that incubation with doxorubicin at a concentration of 3.15 nM initially increases the rate of all the parameters in comparison to control however, they decrease in a time-dependent manner and the rate parameters $k_{xy}$ and $k_{\Sigma A}$ actually are below that of the control by day 21. The rate parameters $k_{x\Sigma}$, $k_{yz}$ and $k_{zx}$ remain above the control for the duration of the experiment. It appears that the rate parameter $k_{x\Sigma}$, in line with what was found with RHPS4, displays the biggest increase above the control, and although there is a decrease over the 21 days of the experiment, the increase above the control is maintained. 3.15 nM was the lowest concentration of doxorubicin analysed and it appears that the biggest rate of change was observed in the rate parameter $k_{\Sigma A}$. At day 4 this rate parameter is 72.6% above the control and as with the other rate parameters, decreases in a time-dependent manner towards the control and by day 21 has reached 53.2% below that of the control. The rate parameter $k_{yz}$ displays the

lowest rate of change reaching a maximum of 17% above the control at day 4, which by day 21 of the experiment has reduced to just 7% above the control.

Increasing the concentration of doxorubicin to 6.3 nM increases the rate of $k_{x\Sigma}$ to 238% above the control; however, unlike doxorubicin at a concentration of 3.15 nM, this increase is maintained for the duration of the experiment and by day 21 has reached a maximum of 580% above the control. There is also a reduction if the rate parameter $k_{\Sigma A}$, however, is not concentration- or time-dependent and decreases to approximately 20% below the control by day 4 and is maintained for the duration of the experiment. Unlike with doxorubicin at a concentration of 3.15 nM, at the higher concentration of 6.3 nM, the rate of $k_{xy}$ is increased by 85% by day 4 and increases in a time-dependent manner reaching 300% above the control by day 21. The rate parameters $k_{yz}$ and $k_{zx}$ appear to not change from the control over the duration of the 21 days.

Increasing the concentration of doxorubicin to 31.5 nM reduces the rate parameters by day 4 to at least 50% below that of the control. The only exception to this is observed with the rate parameter $k_{x\Sigma}$, which increases to approximately 49% above the control by day 4 and increases in a time-dependent manner to 378% above the control by day 21. The rate parameters $k_{zx}$ and $k_{yz}$ remain below the control; however both slightly increase to approximately 5% above the control cells by day 21. The rate parameter $k_{\Sigma A}$ is also observed to be below that of the control, however, this decrease is maintained throughout the duration of the experiment.

The highest concentration of doxorubicin used was 63 nM and at this concentration, the rate of $k_{x\Sigma}$ is again increased to 150% above the control by day 4 and as with the other concentrations, there is a time-dependent increase and reaches a maximum of 272% above the control by day 21 of the experiment. The rate parameter $k_{\Sigma A}$ also remains approximately 60% below the control for the duration of the experiment. It is also evident that the rates of $k_{xy}$, $k_{yz}$ and $k_{zx}$ are reduced below the control by day 4, after which they each increase in a time-dependent manner and are approximately 10% above the control by day 21.

## 5.6 Conclusions

In the laboratory, we are able to easily derive information regarding the effects of RHPS4, and other anti-tumour agents such as doxorubicin on the cell cycle, population doublings and proportion of cells in senescence. Based on the conclusions and deliberations outlined in chapters 2, 3 and 4, it was decided that the best way forward with respect to analysing the cell cycle appears to be that manual method for the analysis of the cycling compartment, i.e. $G_1/G_0$, S and $G_2$/M. For the analysis of the pre-$G_1$ compartment, the most accurate method appears to be using the software packages winMDI and Cylchred. To use these software packages allows the calculation of the pre-$G_1$ proportion based in DNA intensity and therefore reduces any inaccuracies involved with the manual method in which one event is equal to one cell. This assumption is fine for the analysis of the cycling proportion, however, is inaccurate for the analysis of the pre-$G_1$ portion, which as stated previously contains not only apoptotic cells, but also cell fragments, mechanically damaged cells and cells with reduced chromosome number and so for this compartment, one event is not equivalent to one cell. In obtaining population doublings data, we are able to determine how these anti-tumour agents affect the overall growth rate. In addition to this, staining for senescence using the SA-β-gal method as described in chapter 2, allows the determination of how the proportion of senescence cells is affected by RHPS4 and other anti-tumour agents. The cell cycle data allows the proportion of cells in each phase to be analysed and how these are affected. What is not obvious from the data that is derived is how the rate of cells moving from one phase of the cell cycle to the other is affected and the parameters relate much more closely to the actual protein/molecular targets of these drugs than do the emergent properties such as cell cycle distribution. The mathematical models aim to resolve this. The data was obtained experimentally using a range of concentrations of RHPS4 determined from the $GI_{50}$ value over a period of 21 days whereby the cells were collected and analysed at days 4, 6, 10, 14 and 21. The data was then applied to

the models as shown in this chapter to determine the concentration- and time-dependent effects.

The data obtained from RHPS4 was applied to each of the different mathematical models in this chapter to illustrate that depending on which model the data is applied to, the conclusions derived can be very different.

Application of the data to the two-compartment model allowed information regarding the effects on the rate of transition of cells from $G_1/G_0$ and S to $G_2$/M to be determined. The data shown in figure 5.4 depicts that $k_{yx}$ changes over the 21 days of the experiment in a concentration-dependent manner and up to day 10 of the experiment, remains below that of the control, after which incubation with the highest concentration of RHPS4 maintains $k_{yx}$ below the control. Incubation with RHPS4 at concentrations of 50 nM and 100 nM increases $k_{yx}$ above the control in a concentration-dependent manner. The changes in $k_{xy}$ are not as clear and are also shown in figure 5.4. Incubation of the cells with 1 μM RHPS4 reduces the rate of $k_{xy}$ for the duration of the experiment. Again, at concentrations of 50 nM and 100 nM, there is an initial reduction followed by an increase, however, does not occur in a concentration-dependent manner. Application of the data to the two-compartment model does not reveal any useful conclusions and offers no more information that cannot already be derived from the data alone and the behaviour of the apparent rate constants makes little biological sense.

Application of the data to the three-compartment model allows information regarding the effects on the rate of transition of cells from $G_1/G_0$ to S to $G_2$/M to be determined. The graphs in figure 5.5 depict the changes that occur in the rate parameters of the three-compartment model and show that $k_{xy}$ is reduced below the control in a concentration-dependent manner for the duration of the experiment. This is different to the conclusions drawn for this rate parameter with respect to the two-compartment model, however, it must be remembered that for the two-compartment model the rate parameter $k_{xy}$ incorporates the movement of cells from $G_1/G_0$ and S to $G_2$/M, whereas with respect to the three-compartment model, the rate parameter $k_{xy}$ incorporates the movement of cells from $G_1/G_0$ into S. It appears that for $k_{yz}$ and $k_{zx}$ (which exist within the three-compartment model and

not the two-compartment model) the effects of 1 μM RHPS4 appear to reduce both $k_{yz}$ and $k_{zx}$ over the duration of the experiment. It also appears that at 50 nM RHPS4 there is a time-dependent increase above the control which does not appear with 100 nM RHPS4. At a concentration of RHPS4 at 50 nM, there is an initial decrease below the control for both $k_{yz}$ and $k_{zx}$, which by day 6, both rate parameters increase above the control and remain so for the duration of the experiment. When incubated with RHPS4 at 100 nM, there is an initial decrease in $k_{yz}$ followed by an increase before finally decreasing again by day 21, however, consistently remains above the control. The same is true for $k_{zx}$ which appears below the control at the start of the experiment and increases above by day 10 before reducing again. The conclusions are inconsistent with the two-compartment model, and again the behaviour of the apparent rate constants is difficult to explain in a biologically believable way.

Application of the same data to the five-compartment model allows information regarding the effects on the rate of transition of cells from $G_1/G_0$ phase to S phase to $G_2$/M phase before returning to $G_1/G_0$ phase. In addition to these transition rates, the effects on the rate of transition of cells from $G_1/G_0$ phase to senescence and from here to cell death are also analysed in the five-compartment model. It is obvious that in reality, cell death can occur from any stage of the cell cycle if conditions at that point are unfavourable, however, for the purposes of keeping the mathematical model simple, and indeed soluble as we have only five independent variables, and therefore we can only evaluate five rate parameters, we have assumed that the rate of cell death from senescence is much higher than the rate at which it is occurring from $G_1/G_0$, S and $G_2$/M. As this is the case, the rates of cell death from these compartments are not taken into account. For each concentration of RHPS4 analysed, there is a time-dependent increase in $k_{x\Sigma}$ in comparison to control, however, does not appear to be concentration-dependent. In addition to this, there is a decrease in $k_{\Sigma A}$ in comparison to the control which remains so for the duration of the experiment leading to the conclusion that there is an increase in the rate of cells becoming senescent, however, a reduction in the rate at which the cells undergo cell death. To support these conclusions is the evidence that there is

an overall concentration-dependent increase in the proportion of cells in Σ and A collectively which is greater than the proportion of cells in X, Y and Z collectively. This is interesting as there is no concentration-dependent effect on $k_{x\Sigma}$. Analysis of compartments Σ and A individually also reveals that there is an overall increase in the proportion of cells in Σ complemented by a reduction in the proportion of cells in A. Observation of the other rate parameters reveals a slight increase in both $k_{xy}$ and $k_{zx}$ when incubated with 50 nM and 100 nM RHPS4. There appears to be no change in the rate parameter $k_{yz}$ in comparison to control cells. When incubated with RHPS4 at a concentration of 1 μM there is a slight reduction in $k_{xy}$, $k_{yz}$ and $k_{zx}$ suggesting that the cells are able to cycle through $G_1/G_0$ to S to $G_2$/M at a slower rate than normal and are entering senescence at a much higher rate than in comparison to control.

When applied to the mathematical models, the data reveal aspects of the mechanism of action of RHPS4 that would be difficult to appreciate without it. The original data obtained from the flow cytometer and from SA-β-gal stain for senescence clearly shows how the proportions of cells changes in the cell cycle with respect to concentration of RHPS4 and time. What is not obvious from the data is how the kinetics of the cell cycle change and the mathematical model aimed to resolve this.

The purpose of applying the same type of data obtained from doxorubicin, as that what was obtained from RHPS4, was to observe whether the mathematical model can distinguish between the two anti-tumour agents and derive different conclusions. The chemical structure of doxorubicin is very different from that of RHPS4 and both are compared in figure 5.12. In addition, the known mechanism of action of doxorubicin is different from that what is already known of RHPS4. Doxorubicin, also known as adriamycin, is an anthracycline anti-tumour agent and is a known DNA intercalator and there is evidence to show that in cultured mammalian cells there is inhibition of DNA and RNA synthesis producing single-stranded DNA breakage. There is also inhibition of the nuclear enzyme DNA topoisomerase II (Patel *et al*, 1997). There is some overlap with regards to the mechanism of action of RHPS4 and doxorubicin since there is evidence that there

is DNA intercalation with RHPS4. Due to this overlap, there could be similarities in the results of application of the data to the mathematical model.

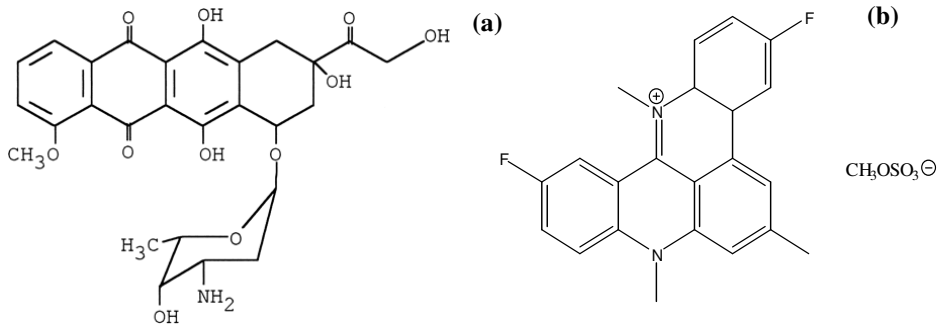



**Figure 5.12 Chemical structures of (a) doxorubicin and (b) RHPS4.** The same data was applied from doxorubicin and RHPS4 in order to determine whether the model could distinguish between the two anti-tumour agents. Doxorubicin has a different mechanism of action to RHPS4 and applying both sets of data to the model allows the comparison of both molecules (Taken from Patel *et al*, 1997; Cookson *et al*, 2005).

The mechanism of action of doxorubicin is very different to that of what is already known regarding RHPS4 and so if upon application of the data to the mathematical model the conclusions are the same, then the five-compartment mathematical model fails to distinguish between the individual anti-tumour agents. Application of doxorubicin data to the five-compartment model reveals that in a very similar manner to RHPS4, there are increases in $k_{x\Sigma}$, however, not to the same degree. This is supported in a study carried out by Sliwinska *et al.* In this study it was found that in response to low doses of doxorubicin in HCT116 cells there is induction of senescence (Sliwinska *et al*, 2008). There is also a reduction in $k_{\Sigma A}$, which is again a reflection of what occurs upon application of RHPS4 data to the model. In addition to these similarities, there are slight increases in $k_{xy}$ with both doxorubicin and RHPS4 with respect to control. At the higher concentrations (31.5 nM and 63 nM) of doxorubicin, there is an overall reduction of $k_{xy}$, $k_{yz}$ and $k_{zx}$ below the control, however, is recovered towards control levels by day 21. Regardless of the similarities observed, we can conclude that the five-compartment mathematical model distinguished between RHPS4 and doxorubicin as shown in figures 5.4 and 5.11. The model derived that RHPS4 affected $k_{x\Sigma}$ to the greatest degree and remained above the control for the duration of the

experiment and increased in a time-dependent manner. Whilst the same occurred for doxorubicin, the increase did not occur to the same degree, and also occurred at a later time point for the higher concentrations of doxorubicin of 31.5 nM and 63 nM. It is also apparent in figure 5.11 that by day 4 of the experiment there are reductions in each of the rate parameters below the control at the higher concentrations analysed, with the exception of the rate parameter $k_{x\Sigma}$. The value of the rate parameters recover over the 21 days eventually reaching values above the control by day 21.

The mathematical models derived in chapter 4 and applied here are very simple mathematical models and whilst they do not fully elucidate the mechanism of action of RHPS4, they go some way in providing insight into what may be occurring at the cellular level. Armed with this information, which would be difficult to appreciate in the absence of the mathematical model, allows further experimental analysis of RHPS4 to be more focused. In addition to providing the insight for further experimental analysis, the models also clearly show how complex RHPS4 is and exposure of the cells to this molecule creates rapid onset effects in HCT116 cells, but there are also effects which take longer to establish.

In conclusion, we are able to develop mathematical models of differing complexities that incorporate as much information of figure 4.1 as shown in chapter 4 as possible. The application of data obtained from RHPS4 to the different mathematical models shows that the conclusions can be very different depending on the mathematical model that is used.

In the next chapter, a software package, COPASI, is used to essentially 'close the loop' and we use the rate constants derived from the mathematical model to simulate the behaviour of the system, and compare its behaviour with the original experimental behaviour.