Chapter 2

Biological Methods and Materials

2.1 Suppliers of Reagents

Abcam plc, Cambridge, UK
Rat monoclonal [HTA28] antibody to Histone H3 (phospho S28), Rabbit polyclonal antibody to Rat IgG H&L (FITC)

Beckman Coulter (UK) Ltd., Buckinghamshire, UK
FlowCheck® beads

British Drug House (BDH), Poole, UK
Glycine

DakoCytomation, Cambridgeshire, UK
monoclonal anti-human Ki-67 antigen (clone MIB-1), rabbit serum (normal), rat serum (normal), polyclonal goat Anti-mouse immunoglobulins/FITC goat $F(ab')_2$, polyclonal rabbit Anti-mouse immunoglobulins/RPE rabbit $F(ab)_2$

Fisher Scientific UK Ltd., Leicestershire, UK
Formaldehyde, gluteraldehyde, hydrochloric acid (HCl), industrial methylated spirits (IMS), methanol (MeOH) (analytical reagent grade), N,N-Dimethylformamide (DMF)

Pharminox Ltd, Nottingham, UK
3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl] acridinium methosulfate (RHPS4) (a gift)

Sigma-Aldrich Co. Ltd., Dorset, UK
bovine serum albumin (BSA), BrdU, citric acid, dimethylsulphoxide (DMSO), doxorubicin, foetal bovine serum (FBS), Ribonuclease A from bovine pancreas (RNAse A), RPMI 1640 liquid medium (containing 0.3 g/L L-glutamine and 2 g/L sodium bicarbonate), monoclonal anti-BrdU clone BU33 (produced in mouse), magnesium chloride, 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), paraformaldehyde (PFA), phosphate buffered saline (PBS) tablets, polyoxyethylenesorbitan (Tween-20), potassium ferricyanide (III), potassium ferrocyanide, propidium iodide (PI) (HPLC grade), saponin, sodium chloride (analytical reagent grade), sodium citrate, sodium phosphate, sodium tetraborate,

Triton X-100, trypsin-EDTA 1x solution, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal),

2.2 Methods

2.2.1 General Cell Culture

All cell culture techniques were carried out aseptically in a BioMat^2 MDH Class II microbiological safety cabinet with a laminar flow system. Before use, the safety cabinet was wiped down with 70% IMS in dH_2O. Colorectal cells (KM12, HCC-2998, HCT116 and HT-29) were sub-cultured twice weekly, or when approximately 70% confluent in order to maintain their exponential growth. The cells were sub-cultured in Costar tissue flasks (25 cm^2 and 75 cm^2) with RPMI 1640 medium (containing 0.3 g/L L-glutamine and 2 g/L sodium bicarbonate), with additional 10% heat-inactivated FBS. FBS was heat inactivated by heating to 55 - 59 °C for 1 hour, and cooled before addition to RPMI tissue culture media. The sub-culturing of cells involved the aspiration of RPMI tissue culture media and the addition of trypsin – EDTA 1x solution to detach the cells from the flask. The cells were then resuspended in fresh RPMI tissue culture media in a new flask at ratios between 1:4 and 1:10, depending on the cell line. Cells were incubated in a LEEC incubator at 37 °C (supplied with CO_2 for a 5% atmosphere and containing a tray of dH_2O to provide a humidified environment).

2.2.2 Cell Stocks

To maintain cell stocks in the cell bank, cells were grown to approximately 70% confluence in 75 cm^2 Costar tissue culture flasks. The RPMI tissue culture media was aspirated and the cells rinsed briefly with sterile PBS and trypsinised as in section 2.2.1. The cells were resuspended in 5 mL of a 5% DMSO/95% heat inactivated FBS solution. The suspension was divided into 1 mL aliquots in cryovials (Nalgene, 1.2 mL) and immediately stored at -20 °C for 24 hours before transfer to -80 °C for a further 24 hours before long term storage at -180 °C.

Upon removal from the -180 °C freezer, the cells were immediately thawed at 37 °C in a water bath. Upon thawing, the cells were added to a single 25 cm^2 flask

containing RPMI tissue culture media (already warmed to 37 °C). The cells were sub-cultured twice before use in assays.

2.2.3 Harvesting Cells

To harvest the cells, all RPMI tissue culture media was aspirated from the flasks and briefly rinsed with sterile PBS. Cells were detached from the flask with 500 µL trypsin-EDTA per 25 cm^2 and re-suspended in 10 mL PBS. To pellet the cells, they were centrifuged at 1200 rpm for 5 minutes and the supernatant removed. The cell pellet was resuspended in 20 mL PBS and centrifugation repeated to yield a final cell pellet which was stored at -80 °C.

2.2.4 Cell Lines

The HCC-2998 cell line is a highly differentiated human colon carcinoma cell line (Shi *et al*, 2006). It was originally isolated from a carcinoma of the colon and cryopreserved in 1989 and has an adherent mode of growth. The doubling time is 31.5 hours (http://dtp.cancer.gov/docs/misc/common_files/cell_list.html-accessed 11/11/2008).

The KM12 cell line originated from an adenocarcinoma of the colon cryopreserved in 1989. KM12 cells also have an adherent mode of growth; however, have a doubling time of 23.7 hours (Morikawa *et al*, 1988).

The HT-29 cell line originated from a human colon adenocarcinoma established from the primary tumour of a 44-year-old Caucasian woman with colon adenocarcinoma in 1964. The HT-29 cell line, also with an adherent mode of growth, has a doubling time of 19.5 hours (Fogh & Trempe, 1975).

The HCT116 cell line is one of 3 strains of malignant cells isolated from a male with colonic carcinoma. HCT116 cells have an adherent mode of growth and have a relatively short doubling time of 17.4 hours (Brattain *et al*, 1981).

2.2.5 Drug/Agent Stocks

All stocks were made up to a concentration of 10 mM in DMSO and stored at 4 °C and protected from light. Stocks were used for a maximum of 12 weeks and prior to use were sterile filtered using a 0.2 µM Sartorius sterile filter.

2.2.6 MTT Assay for Cell Viability

The method employed for the MTT assay was adapted from Mosmann (1983). The cells were harvested as in section 2.2.3, however were not frozen and instead were syringed through a 23G needle to obtain a near-single suspension. The cells were counted using a haemocytometer and seeded at the desired density of cells per well of a 96-well plate. Due to the differing growth properties of each cell line, each was seeded at slightly different densities as shown in table 2.1 in 180 μL RPMI tissue culture media per well. The wells of the outer columns of the 96-well plate were filled with 300 μL RPMI tissue culture media alone to prevent evaporation from the experimental wells and also provide blanks for the plate reader. The cells were incubated overnight at 37 °C in a 5% CO_2 humid environment to allow the cells to attach. A time zero (t0) plate was also prepared, treated in the same way as the other plates, however was used as a measure of cell viability at the time the agent was added. Following attachment, serial dilutions were made in RPMI tissue culture media and 20 μL was added to each well to give final concentrations of the agent in the well of 300 μM, 100 μM, 30 μM, 10 μM, 3 μM, 100 nM, 10 nM and 1 nM. RPMI tissue culture media (20 μL) was added to a single column of cells as a control.

Treated cells were incubated between 24 and 72 hours at 37 °C in a 5% CO_2 atmosphere. After the appropriate length of time, cell viability was quantified. To do this, 50 μL of sterile filtered (0.2 μM Sartorius sterile filter) MTT (concentration of 2 mg/mL in PBS) was added to each well, giving a final concentration of 0.4 mg/mL and the cells re-incubated for 4 hours. Cells which were viable were able to convert MTT into purple formazan crystals by mitochondrial dehydrogenases and therefore by quantifying the amount of dye formed; there is an indirect quantification of the number of viable cells remaining following treatment with agent is obtained. MTT treated cells were incubated at 37 °C for 4 hours, after which the drug/RPMI tissue culture media from each well was aspirated and the formazan product dissolved in 150 μL DMSO- glycine buffer (0.1 M glycine, 0.1 M NaCl, pH10) at a ratio of 4:1. The cells were placed on an orbital plate shaker in order to aid the dissolving of the formazan product.

The plate was read on a photometric plate reader at 550 nm using an Anthos Labtec systems plate reader with Deltasoft 3^{TM} software. Resultant growth curves were constructed using Microsoft Excel. Log drug concentration plotted versus A_{550} allowed GI_{50} values to be calculated. MTT assays were repeated a minimum of three times and the GI_{50} values were calculated as the average of these.

2.2.7 Cell Cycle Analysis

2.2.7.1 General Cell Cycle Analysis

HCT116, HT-29, KM12 and HCC-2998 cells were grown in 25 cm^2 flasks in a total volume of 8 mL of RPMI tissue culture medium, including agent (except for controls) and maintained at 37 ^{o}C in a 5% CO_2, humidified atmosphere until preparation for analysis. The cell densities used for the individual cells lines are shown in table 2.1. Cells grew at different rates according to agent concentration and the duration, for which they were exposed to drug, therefore the cells were passaged when they were approaching 70-80% confluence, at a density between 1 in 2 and 1 in 10, according to the anticipated doubling rate and agent concentration. Fresh medium and agents, where appropriate, were replaced every 3-4 days. Analyses were always performed on cells no more than 60-70% confluent, to maximise the proportion of cycling cells. Higher initial seeding densities were used for the 24 hour, 48 hour and 72 hour analyses to compensate for reduced population doublings.

The protocol used for the cell cycle analysis of colorectal cell lines was adapted from Nicoletti *et al* (1991). The cells were harvested as in section 2.2.3 and counted using a haemocytometer. The cells were resuspended in PBS at a density of $2x10^5$ cells in a FACS tube where they were centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatant was decanted and the pellet resuspended in 400 µL hypotonic fluorochrome solution (as shown in table 2.2). The cells were kept at 4 °C for 24 hours in the dark prior to analysis in which the PI fluorescence of individual nuclei was measured using a Coulter Epics XL-MCL™ flow cytometer operated using Expo32™ software. Before using the Coulter Epics XL-

MCL™ flow cytometer, a quality control check was carried out according to the manufacturer's instructions to monitor instrument alignment using FlowCheck® beads. When the quality control check was satisfactory, the cells were analysed. List mode data for forward scatter-height (FSC-H), side scatter-height (SSC-H) and fluorescence emission in the FL3 channel (representing PI fluorescence) of particles were collected. A threshold value of 30 was set for collection in the FL3 channel as a discriminator for cell debris. The flow rate was set to low and a minimum of 20 000 events were analysed. The proportion of cells in each phase of the cell cycle (pre-G_1, G_1/G_0, S and G_2/M) was obtained by setting gates manually on the DNA content histograms obtained from FL3 fluorescence, using Expo32™ software.

Table 2.1 Cell seeding densities used in MTT assay for cell viability and cell cycle analysis

Cell Line	**Basic cell cycle analysis**		**MTT assay**	
	cells/well			
	24-48 hour	72-144 hour	24 hour	72 hour
HCT116	30 000	25 000	5000	2000
HT-29	30 000	25 000	5000	2000
HCC-2998	35 000	30 000	5000	2500
KM12	35 000	30 000	5000	2500

Table 2.2 Composition of hypotonic fluorochrome solution used in cell cycle analysis

Fluorochrome solution component	**Strength (final)** [1]
Sodium citrate	0.1%
Triton-X-100	0.1%
PI	50 µg/mL
RNAse A	0.1 mg/mL

[1] *in distilled water*

2.2.7.2 Data Extraction for a Systems Biology Approach

Data extraction for a systems biology approach has to be carried out in a particular manner to allow the achievement of a systematic analysis of the experimental cytometry data. The data has to be extracted in a regimented manner that allows it to meet the needs of a systems biology approach. Below is a description of the data processing that occurred in order to derive the proportions of cells in G_1/G_0, S and G_2/M from a population of cells using flow cytometric analysis that we can

use in our mathematical model. The method used was carried out as Nicoletti *et al*, 1991 and in addition to allowing the identification of the proportions of cells in G_1/G_0, S and G_2/M, a pre-G_1 fraction is also identified, which is representative of cell degradation products, 'dead' cells, which may or may not be apoptotic, mechanically damaged cells, cells with reduced chromosome number and isolated apoptotic bodies. Apoptosis can also result in the complete fragmentation of the nucleus and cytoplasm and when staining of the cells with a hypotonic buffer, several individual nuclear fractions can be produced from a single apoptotic cell and can therefore result in the over-estimation of the proportion of apoptotic cells. Shown here is the derivation of the proportion of cells in G_1/G_0, S and G_2/M. The cells were harvested as in section 2.2.3 and incubated in hypotonic fluorochrome solution as described in section 2.2.7.1. The flow cytometer was instructed to acquire the data as follows:

1. A dot plot of FSC-H versus SSC-H allows us to gain an estimation of the viable fraction.
2. A dot plot of FL3-lin versus AUX from the viable cells that have been gated in (1) allows us to 'pull out' the single cells away from the unwanted doublets.
3. A dot plot of FSC-H versus FL3-Lin of the viable cells allows the further gate out debris.
4. A 'clean' histogram (< 20 000 events) of FL3-lin of the cells gated in (1) and (2) is used to determine the proportion of cells in G_1/G_0, S and G_2/M. To do this, a fitting algorithm is used based on Watson *et al*, 1987.

In carrying out the above procedure on each set of data and placing gates (1) and (2), we can gate out the cell debris and doublets and therefore allow the histogram of the cell cycle to be 'clean'. Markers allow us to segment the histogram and are placed to allow manual estimations of the proportions of cells in G_1/G_0, S and G_2/M. In using manual estimations, objectivity is difficult to achieve and therefore rules for the segmenting of the histograms needs to be in place. An example of the data extraction used in this study is shown in appendix I.

2.2.7.3 BrdU Analysis for G_1 – S Phase Kinetics

BrdU analysis allows the proportion of cells in S phase to be accurately analysed. For analysis, the cells were analysed when 60-70% confluent to ensure maximum collection of cycling cells. Cells were grown in 25 cm^2 Costar tissue culture flasks in a total volume of 8 mL of RPMI tissue culture medium, including agent (except for control) and maintained at 37 ^oC in a 5% CO_2, humidified atmosphere until preparation for analysis. When required, the RPMI tissue culture medium was aspirated and 15 μM BrdU (volume of 10 mL/25 cm^2) was added to the cells. Cells were incubated at 37 ^oC in a 5% CO_2, humidified atmosphere for 2.5 hours and following incubation, the RPMI tissue culture media was aspirated and the cells washed twice in fresh RPMI tissue culture medium. The cells were harvested and resuspended in 5 mL RPMI tissue culture medium and counted, again using a heamocytometer. Cells were resuspended at a density of $1x10^6$ cells in FACS tubes and centrifuged at 1200 rpm for 5 minutes at 4 °C and the supernatant decanted (see table 2.4 for conditions adopted for the BrdU incorporation assay). The cells were resuspended in 5 mL PBS and vortexed prior to centrifugation at 1200 rpm for 5 minutes at 4 °C. This step was repeated twice. Following the second centrifugation, cells were fixed by breaking the pellet through vortexing whilst adding 5 mL ice-cold 75% MeOH. The cells were left for a minimum of 2 hours at 4 °C and were disturbed occasionally, again by vortexing. For long term storage prior to analysis, the samples were stored at -20 °C for up to 2 weeks. Following incubation, the cells were thawed and centrifuged at 1200 rpm for 5 minutes and the supernatant decanted. Vortexing the pellet allowed it to be thoroughly broken and was then washed twice in 5 mL PBS (each time, centrifuging at 1200 rpm for 5 minutes at 4 °C, decanting the supernatant and resuspending in 5 mL PBS). The cells were incubated at room temperature (RT) for 30 minutes in 2 M HCl and centrifuged again at 1200 rpm for 5 minutes at 4 °C and the supernatant discarded. This step was repeated twice. The cells were resuspended in wash buffer (for composition, see table 2.4) and centrifuged, again at 1200 rpm for 5 minutes at 4 °C. Following the final centrifugation, the samples were resuspended in 1 mL 0.1 M sodium tetraborate (see table 2.3) and incubated

at RT for 2 minutes. The cells were resuspended in 1 mL wash buffer and centrifuged at 1200 rpm for 5 minutes at 4 °C. This step was repeated twice and following the final centrifugation, the cells were incubated in monoclonal anti-BrdU clone BU33 (produced in mouse) (for dilutions and volumes, see table 2.4) for 1 hour at RT. Following incubation with monoclonal anti-BrdU clone BU33, the cells were resuspended in 1 mL wash buffer and centrifuged at 1200 rpm for 5 minutes at 4°C. This step was repeated 3 times. The cells were incubated in polyclonal goat anti-mouse immunoglobulins/FITC/ goat $F(ab')_2$ (for dilutions and volumes, see table 2.4) and were again incubated for 1 hour at RT in the dark. The cells were again washed 3 times in 1 mL wash buffer. Following the final wash, the cells were resuspended in 500 µL PBS with 10 µg/mL PI and 100 µg/mL RNase A.

Each sample was syringed through a 23G needle to separate cells prior to analysis using the Coulter Epics XL-MCL™ flow cytometer. List mode data for forward scatter, side scatter, and fluorescence emission in the FL1 and FL3 channel (representing BrdU and PI fluorescence, respectively) of particles was collected. A threshold value of 30 was set for collection in the FL3 channel as a discriminator for cell debris and the flow rate was set to low and minimums of 20 000 events were analysed.

2.2.7.4 Determining Mitotic Population with Histone Immunofluorescence

Histone H3 immunofluorescence allows accurate analysis of the proportion of mitotic cells to be separated from the proportion of G_2 cells within a population using a flow cytometer. Cells were cultured and treated in 25 cm^2 Costar tissue culture flasks in a total volume of 8 mL of RPMI tissue culture medium, including agent (except for control) and maintained at 37 ^oC in a 5% CO_2, humidified atmosphere until preparation for analysis. The cells were harvested as in section 2.2.3, however, not frozen and instead were resuspended in RPMI tissue culture media. The cells were centrifuged at 1200 rpm for 5 minutes at 4 °C, and the supernatant was decanted. The cells were fixed by vortexing to break the pellet whilst ice-cold 75% MeOH was added. The cells were aliquoted at a density of

$2x10^5$ cells. In addition to the samples of treated and untreated, there was also an unstained control sample (for autofluorescence). The cells should be washed in 1 mL PBS/1% BSA (PBSA) (see table 2.3) and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 1 mL 2% PFA (see table 2.3). The samples were mixed well and left at RT for 10 minutes. Following this incubation, the samples were resuspended in PBSA (see table 2.3) and centrifuged at 1200 rpm for 5 minutes at 4 °C the supernatant discarded. This step was repeated twice. Following the final centrifugation, the cells were resuspended in 100 µL 0.1% saponin buffer (see table 2.3) and incubated at RT for 10 minutes. Following this incubation, the cells were resuspended in 1 mL PBSA/0.2% rat serum and centrifuged at 1200 rpm for 5 minutes at 4 °C. The supernatant was discarded and resuspended in 1 mL PBSA/0.2% rat serum and again centrifuged at 1200 rpm for 5 minutes at 4 °C and was repeated three times. Following the final centrifugation, the supernatant was discarded and the pellet was vortexed and resuspended in rabbit monoclonal [HTA28] to Histone H3 (phospho S28) (for dilutions and volumes, see table 2.4). The cells were incubated for 1 hour at RT in the dark, disturbing by vortexing occasionally. Following incubation, 2 mL PBS/1% BSA/0.5% Tween-20 (PBSAT) (see table 2.3) was added to the cells and vortexed gently on and off for 5 minutes. The samples were centrifuged, again at 1200 rpm for 5 minutes at 4 °C and the supernatant discarded. This step was repeated three times, each time resuspending in PBSAT followed by centrifugation. Following the three washes, rabbit polyclonal to rat IgG H&L (FITC)/0.2% rat serum (for dilutions and volumes, see table 2.4) was added directly to the cells and incubated for 1 hour at RT in the dark and vortexed occasionally. Following incubation, 2 mL PBSAT was added to the cells and vortexed gently on and off for 5 minutes before centrifuging at 1200 rpm for 5 minutes at 4 °C. This step was repeated twice before resuspending the pellet in 500 µL PBSA.

Each sample was syringed through a 23G needle to separate cells prior to analysis using the Coulter Epics XL-MCL™ flow cytometer. List mode data for forward scatter, side scatter, and fluorescence emission in the FL1 and FL3 channel

(representing histone H3 S28 and PI fluorescence, respectively) of particles was collected. A threshold value of 30 was set for collection in the FL3 channel as a discriminator for cell debris. The flow rate was set to low and a minimum of 20 000 events were analysed.

2.2.7.5 Determining G_0 Population with Ki-67 Immunofluorescence

Ki-67 allows the proportion of proliferating cells to be accurately accounted for and therefore separating the G_0 cells from proliferating cells on the flow cytometer. Cells were cultured in 25 cm^2 Costar flasks in a total volume of 8 mL of RPMI tissue culture medium, including agent (except for control) and maintained at 37 ^oC in a 5% CO_2, humidified atmosphere until preparation for analysis. The RPMI tissue culture medium was aspirated, trypsin was added to the cells in the tissue culture flask and incubated for 3-5 minutes at 37 °C and the cells were resuspended in 5 mL RPMI tissue culture media. The cells were centrifuged at 1200 rpm for 5 minutes at 4 °C and the supernatant decanted. The cells were resuspended in PBS and again centrifuged at 1200 rpm for 5 minutes at 4 °C. Following this step, the supernatant was decanted and the pelleted cells were vortexed thoroughly whilst 5 mL ice-cold 75% MeOH was added. The cells were incubated overnight at 4 °C in the dark. Following the fixing step, the cells were centrifuged at 1200 rpm for 5 minutes at 4 °C and the supernatant discarded before being resuspended in 5 mL PBSA (see table 2.4). The cells were centrifuged, again at 1200 rpm for 5 minutes at 4 °C and the supernatant discarded and. This step was repeated twice after which the cells were aliquoted at a density of $2x10^5$ cells per sample as required. It was also necessary to have an autofluorescence sample. The cells were incubated in monoclonal anti-human Ki-67 antigen (clone MIB-1), (for dilutions and volumes, see table 2.4) for 1 hour at RT in the dark, disturbing occasionally. Following incubation, the cells were centrifuged at 1200 rpm for 5 minutes at 4 °C and resuspended in 1 mL PBSAT. This step was repeated twice. Following the final centrifugation, the cells were incubated in polyclonal rabbit anti-mouse immunoglobulins/RPE rabbit $F(ab')_2$/1% rabbit serum (for dilutions and volumes, see table 2.4). The cells were left for 1 hour in

the dark at RT, disturbing occasionally, following which the cells were centrifuged at 1200 rpm for 5 minutes at 4 °C and the supernatant discarded. This step was repeated twice. Following the final centrifugation, the cells were resuspended in 500 µL PBS/500 µg/mL PI/0.1 mg/mL RNAse A and incubated on ice for 10 minutes.

Each sample was syringed through a 23G needle to separate cells prior to analysis using the Coulter Epics XL-MCL™ flow cytometer. List mode data for forward scatter, side scatter, and fluorescence emission in the FL2 and FL3 channel (representing Ki-67 antigen and PI fluorescence, respectively) of particles was collected. A threshold value of 30 was set for collection in the FL3 channel as a discriminator for cell debris. The flow rate was set to low and minimums of 20,000 events were analysed.

Table 2.3 Components of solutions used in the flow cytometric analysis of colorectal cell lines.

Solution	**Component**
Wash Buffer	1% FBS/0.5% Tween 20 in PBS
PBSA	PBS + 1% BSA
PBSAT	PBS + 1% BSA + 0.5% Tween 20
2% PFA	Dissolve 2 g PFA in 50 mL PBS. Heat in water bath (in fume cupboard) at 70 °C for 30 minutes until dissolved. Allow to cool and mix 1 part 4% PFA
0.1% saponin buffer	Dissolve 0.02 g saponin in 20 mL PBS
0.1 M sodium tetraborate	Dissolve 152.8 mg in 75.9 mL dH_2O

Table 2.4 Volumes and dilutions of the antibodies used in the cell cycle analysis of colorectal cell lines

Antibody	**Volume**	**Dilution**
monoclonal anti-BrdU clone BU33	10 µL	1:100
polyclonal goat anti-mouse immunoglobulins/FITC/ goat $F(ab')_2$	10 µL	1:30
rabbit monoclonal [HTA28] to Histone H3 (phospho S28)	2 µL	0.5 mg/mL
rabbit polyclonal to rat IgG H&L (FITC)	10 µL	1:200
monoclonal anti-human Ki-67 antigen	10 µL	1:100
polyclonal rabbit anti-mouse immunoglobulins/RPE rabbit $F(ab')_2$	500 µL	1:200

2.2.8 Staining and Photo-Microscopy for β-galactosidase Activity as an Indicator of Senescence

The SA-β-gal protocol was adapted from Dimri *et al* (1995). Cells were grown in 25 cm^2 Costar tissue culture flasks in RPMI tissue culture medium containing 10%

FBS (controls) and agent. The cells were exposed to agent for up to 21 days and were analysed at days 4, 6, 10, 14 and 21. The different cell lines grew at different rates according to both length of exposure and concentration to agent and therefore were passaged when 60-70% confluent, at a density between 1 in 2 and 1 in 10, according to anticipated doubling rate. Senescence analysis was always performed on sub-confluent cells as over-confluence led to background staining. The media was aspirated from the cells and they were rinsed with PBS and fixed for 10 minutes at RT in fixative solution (2% formaldehyde/0.2% gluteraldehyde in PBS). The cells were rinsed 3 times with PBS and 5 mL stain solution (see table 2.5) was added per flask. All solutions were sterile filtered (0.2 μM Sartorius sterile filter) and stored at 4 °C with the exception of X-gal, which was stored at -20 °C and protected from light. The X-gal was added to a final concentration of 1 mg/mL from a 20 mg/mL solution in DMF prepared immediately before use in a glass container. The flasks were foil-wrapped and incubated at 37 °C overnight (for a minimum of 16 hours) then viewed by phase contrast microscopy (Olympus, Nikon Eclipse T5100) under 10x magnification. Digital images were taken of 5 random fields of view per flask, using a Nikon Coolpix 4500 digital camera and a further zoom factor of 4 was applied.

To quantify the relative staining from the digital photographs, the numbers of stained cells were counted using the software package Jasc® Paint Shop Pro™ 7. For each digital photograph, the total number of cells and the total number of stained cells were counted and the percentage numbers of stained cells were calculated.

Table 2.5 Composition of the senescence marker staining solution

Stain solution component[1]	**Final concentration**
Potassium ferricyanide	5 mM
Potassium ferrocyanide	5 mM
Sodium chloride	150 mM
Magnesium chloride	2 mM
Sodium phosphate/citric acid buffer, pH 6.0	40 mM
X-gal	1 mg/mL

[1]*The stain solution (excluding X-gal) was prepared in distilled water and stored at 4 °C. X-gal was dissolved immediately before use in DMF at 20 mg/mL then added to stain solution at the final concentration of 1 mg/mL.*

2.2.9 Long Term Effects of RHPS4 on Cellular Proliferation

Cells were seeded at a density of $1x10^6$ cells/75 cm^2 in 20 mL RPMI tissue culture media and incubated overnight to allow the cells to attach. The cells were treated with 1 μM RHPS4 and re-incubated at 37 °C in a 5% CO_2 atmosphere. Digital images of the cells were taken on a daily basis for 10 days (allowing small changes to be analysed). Due to the different growth properties of the cells, when the cells had reached 60-70% confluence, they were sub-cultured and the RPMI tissue culture media replaced.

The longer term effects of RHPS4 and doxorubicin on HCT116 cells were also analysed. The cells were seeded at an initial density of $2x10^5$ in 25 cm^2 Costar flasks in 10 mL RPMI tissue culture medium (plus 10% FBS). The appropriate agent was added to the RPMI tissue culture medium at concentrations of 0.1x GI_{50}, 0.5x GI_{50} and 1x GI_{50} and the cells maintained at 37 °C in a humidified 5% CO_2 atmosphere for up to 21 days. The cells were collected and analysed on days 4, 6, 10, 14 and 21. On the day of analysis, the cells were harvested as in section 2.2.3. The cells were counted using a haemocytometer and their cell cycle analysed as in section 2.2.7. The number of population doublings at each time point was also calculated as shown in equation (2.2.9.1).

$$n = (\log P_n - \log P_0)/\log 2 \qquad \textbf{(2.2.9.1)}$$

where P_n is the number of cells after n doublings;

P_0 is the initial seeding density i.e. 2×10^5

The cumulative number of population doublings was plotted against time.

At each time point, the cells were collected and $2x10^5$ cells were placed in fresh RPMI tissue culture medium with the correct concentration of agent. The cells were incubated at 37 °C in a 5% CO_2, humidified atmosphere until the next collection point.