5. *In vitro* antiproliferative bioassays

5.1. Introduction

So as to speed research, decrease development costs and improve therapeutic response, there is an increased interest in designing combination drug regimens rationally, early in the pre-clinical drug development stage. *In vitro* bioassays using 96 well plates is an effective way of screening various drug-drug combinations at specified ratios and various concentrations for cytotoxic activity against different cell cultures.

In contemporary scientific literature, synergy is best defined as greater than the expected additive activity of two or more drugs. Drug interactions (whether synergistic, additive or antagonistic) are dynamic and are dependent upon both the ratio of the drugs and on the dose level. [92] Distinguishing between synergistic, additive and antagonistic drugs combinations requires experimental designs and statistical analysis that require appreciable experimental time and effort. [93] Each individual fixed-ratio combination tested can be considered as a unique drug entity.

Many methods including fractional product analysis, isobologram methodology and surface response models have been put forward to evaluate drug interactions. The most popular analysis technique is the combination index method based on the median effect principle derived from the law of mass action. [94] Median effect analysis allows for the evaluation of drug combinations irrespective of whether the drugs exert their effect in the same pathway or through a completely different mechanism of action. [95] Such analysis is widely used as its application has been simplified by the availability of user-friendly software (CalcuSyn).

The Median-effect equation correlates dose and effect in the simplest form possible:

$$\mathbf{\mathit{f}a / \mathit{f}u = (D / D_m)^m}$$

Where: D: dose of the drug; D_m: median effect dose signifying the potency (similar to IC_{50}); $\mathit{f}a$: the fraction affected by the dose; $\mathit{f}u$: the fraction unaffected;
m: exponent signifying the sigmoidicity (shape) of the dose effect curve

As stated, synergy is defined as a more than the expected additive effect and antagonism as less than the expected additive effect. A combination index (CI) of 1 at a selected fractional affect ($\mathit{f}a$) represents an additive effect (the combined effect of the drugs used separately, results in the same effect as if the drugs were used together at the equivalent doses), while a CI<1 represents synergism and a CI>1 represents antagonism.

A combination index (CI) at a particular fractional affect ($\mathit{f}a$) can be obtained from:

$$\mathbf{CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_1(D)_2/(D_x)_1(D_x)_2}$$

Where: $(D)_1$ and $(D)_2$: represent the dose of the drugs (in combination) eliciting a particular fractional affect and $(D_x)_1$ and $(D_x)_2$ are the doses of the drugs (used alone) that elicit the same fractional affect.

To perform such analysis: dose response curves are required for each of the respective drugs individually and dose response curves are required for each potential FRDC. In this study we elected to combine both B663 and the lead TMP derivative (B4125) with three standard chemotherapeutics (SC): etoposide, paclitaxel and vinblastine. These first-line SC were elected for combination with Riminophenazines for two reasons. Firstly on the basis that these cytotoxic drugs have been reported to be substrates of Pgp (often leading to clinical failure) and secondly that they possess different mechanisms of action and may therefore be used to derive mechanistic information.

Phenotypically stable (intrinsic) Pgp expressing, neoplastic cell cultures (COLO 320DM and HCT-15) were used to evaluate the antiproliferative effect of various fixed molar ratio drug combinations. The purpose of such investigations was to identify what specific ratios and of which drug combinations possess synergy against MDR neoplastic cell cultures.

Additionally, the hypothesis that Riminophenazines could be used as inhibitors of additional ABC transporters was evaluated. To this end, a MRP expressing cell line (ASH-3) was obtained.

5.2. Materials

Cell cultures

COLO 320DM (ATCC number: CCL-220) and HCT-15 (CCL-225), human colorectal carcinoma cell cultures were obtained from American Tissue Culture Collection (ATCC). Both these cultures were grown in Roswell Park Memorial Institute (RPMI) media with 10% Fetal calf serum (FCS).

ASH-3 (JCRB 1073), a human anaplastic thyroid carcinoma cells were purchased from the Japan Health Sciences Foundation (Health Science Research Resources Bank) and cultured in a 1:1 mixture of RPMI and Dulbecco's Modified Eagel's Medium (DMEM) with 10% FCS.

CalcuSyn ver.2.0 software for performing dose effect analysis was purchased from Biosoft (Cambridge, UK).

Preparation of reagents

Ammonium chloride solution

Ammonium chloride solution was prepared by dissolving 8.3 g Ammonium chloride (NH_4Cl), 1 g Sodium bicarbonate ($NaHCO_3$), (both purchased from Merck, JHB,

SA) and 74 mg EDTA (Sigma Aldrich, JHB, SA) in 1000 ml of distilled water. The solution was filter sterilized using a 0.2 µm syringe filter and refrigerated until use

Cell counting fluid

One millilitre of a 0.1% crystal violet solution and 2 ml glacial acetic acid was dissolved in 97 ml of distilled water. The solution was mixed well and refrigerated until used.

Dulbecco's Modified Eagle's Medium (DMEM)

DMEM powder (Sigma-Aldrich, JHB, SA) was dissolved in sterile water with the aid of a sterile stirrer. The pH of the solution was adjusted to 4 with 1 N HCl to ensure complete solubilisation. Thereafter 2 g of $NaHCO_3$ was added to each litre of the medium. The pH was readjusted to 7.1 through the addition of either 1N HCl or 1 N NaOH. The medium was filter-sterilized through a 0.2 µm filter and divided into 500 ml aliquots. Typically, 55 ml was removed from each 500 ml aliquot and the remaining solution was supplemented with 5 ml of a 1% penicillin/ streptomycin solution and 50 ml sterile heat inactivated fetal calf serum (HI FCS). The medium was stored at 4ºC until used.

Heparin

Thirty grams of Heparin (Sigma-Aldrich, JHB, SA) was dissolved into 90 ml of distilled water. The solution was filtered sterilized using a 0.2 µm syringe filter and refrigerated until use.

MTT solution

A stock solution was prepared by dissolving 200 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (Sigma-Aldrich, JHB, SA) in 40 ml PBS solution. After solubilising the light sensitive solution was filter sterilized using a 0.2 µm syringe filter and stored (covered in tin foil) at 4ºC until use.

Phosphate buffered saline (PBS)

Using the manufacturers recommendations, 0.923 g of buffer powder (The Scientific Group, JHB, SA) was dissolved in 100 ml of deionised water. The pH was adjusted to 7.2.

Roswell Park Memorial Institute Medium (RPMI) 1640

RPMI 1640 powder (Sigma-Aldrich, JHB, SA) was dissolved in sterile water with the aid of a sterile stirrer. The pH of the solution was adjusted to 4 with 1N HCl to ensure complete solubilisation. Thereafter 2 g of $NaHCO_3$ was added to each litre of the medium. The pH was readjusted to 7.1 through the addition of either 1N HCl or 1N NaOH. The medium was filter-sterilized through a 0.2 µm filter and divided into 500 ml aliquots. Typically, 55 ml was removed from the 500 ml aliquot and the remaining solution was supplemented with 5 ml of a 1% penicillin/ streptomycin solution and 50 ml sterile HI FCS. The medium was stored at 4ºC until used.

Trypan blue

A 0.2% w/v stock solution was made in PBS.

Standard Chemotherapeutics (SC):

Paclitaxel (PTX), etoposide (ETOP), vinblastine (VIN) and doxorubicin (DOX) were purchased from Sigma Aldrich (St Louis, MO, USA).

Clofazimine was kindly supplied by Dr. J.F. O'Sullivan, Laboratories of the Medical Research Council of Ireland, Trinity College, Dublin, Republic of Ireland.

Water was 18 MΩ water produced from the municipal water supply after processing by an ELGA purification unit (ELGA, Wycombe, UK).

5.3. Methodology

5.3.1. Cell culture preparation and *in vitro* antiproliferative assays

All handling of cultures and experiments were conducted sterile within an ISO particle count certified High Efficiency Particulate Air (HEPA) filtered laminar flow cabinet.

Cultures of the respective cell lines were established using standard *in vitro* methods and the suppliers recommended media in 25 - 175 cm^2 plastic culture flasks (AEC-Amersham P/L, JHB, SA) to achieve the required cell numbers for each assay. Cultures were incubated at 37℃ in a humidified atmosphere with 5% CO_2. The cultures were regularly sub-cultured in order to maintain log phase growth.

On each day of experiment initiation, cells were harvested from their flasks using 0.25% (w/v) trypsin/EDTA with the aid of gentle agitation. Non-adherent COLO 320DM cells were simply decanted into 15 ml tubes and centrifuged at 350 *g* for 5 minutes at room temperature. The cell pellets were resuspended in 1 ml of complete medium and thoroughly aspirated. An aliquot (typically 50 µl) of the cell suspension was taken and added to 450 µl of a trypan blue solution before performing cell counts and assessing viability using a haemocytometer.

The remaining cell suspension was adjusted to a suitable volume using complete media to yield the final desired cells/ml concentration based on previously determined cell line specific seeding numbers for the duration of a particular assay. A volume of 100 µl of the adjusted cell suspension was seeded into 96 well tissue culture plates (Nunc). Cell seeding numbers were as follows: 500 cells/well for COLO 320DM cells, 2500 cells/well for HCT-15 cells and 10000 cells/well for ASH-3 cells. A further 80 µl of the specific complete medium was added to each well. Cultures were pre-incubated for a period of 1 hr for the COLO cell line and 24 h for the HCT and ASH cell line to allow the cells attach. Thereafter, 20 µl of each of the pre-prepared, 10 x concentration of the respective drug concentrations were

pipetted into each of the corresponding experimental wells to make up a total volume to 200 µl thus producing a 1:10 dilution (in well) of the drug concentration. After an incubation period of 7 days for the COLO cell line or 3 days for the HCT and ASH cell lines, MTT cell enumeration assays were performed as follows:

MTT assay (Methods of Mossmann [96] with modifications):

This assay is based on the reduction of yellow MTT into purple formazan crystals by metabolically active cells. This assay was used to quantitate the percentage of viable cells compared to untreated controls.

A volume of 20 µl of a 5 mg/ml MTT solution in PBS was added to each well. Plates were incubated for a further 3 - 4 hours in 5% CO_2 at 37ºC. Plates were then centrifuged at 500 *g* for 10 minutes before the supernatants of each well were carefully removed without disturbing the pellet. Pellets were washed with 150 µl PBS and again the plates were centrifuged at 500 *g* for 10 minutes. The supernatant was again carefully removed before drying the plate overnight. The next day 100 µl of DMSO was added to each well. Plates were gently shaken on a model VRN-200 shaker for at least an hour before they were read spectrophotometrically using a microplate reader (Bio-Tek instruments, ELx 800 UV) at 570 nm, using 630 nm as a reference. The absorbance values were used to determine the relative percentage of viable cells compared to the untreated controls.

5.3.2. Fixed molar ratio drug combination studies

Experimental design and procedures: COLO 320DM

The following stock solutions were made up in DMSO: Riminophenazines (B663 & B4125) - 2 mM; Etoposide - 4 mM; Paclitaxel - 1 mM; Vinblastine - 0.5 mM.

Drug combination studies were performed in a manner similar to that described in the CalcuSyn software manual. As a first step, IC_{50} values were estimated for each

of the drugs alone against COLO neoplastic cell cultures using various concentrations of drug after 7 days exposure, determining cell survival relative to untreated controls then using GraphPad Prism version 4 to plot a response curve from which the individual drug IC_{50} values were estimated. ETOP, PTX and VIN were then combined with either Riminophenazine: B663 or B4125 at the following arbitrary fixed molar ratios: 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10 and tested for drug response over an appropriate concentration range.

To accomplish this, the highest desired concentrations (in well), of a particular ratio for a particular combination (e.g. 0.2:0.4 µM, PTX: B663, 1:2) was multiplied by 10 and by 2 to compensate for the 1:10 dilution in well and for mixing equivalent volumes of each of the drugs making up the FRDC. Appropriate volumes of working solutions for each of the respective FRDC were made up in complete medium from stock solutions. From these working solutions, 8 further calculated serial dilutions were made to cover an expected dose response range.

Experiments were performed in triplicate at each concentration level (Table 5.1.) Absorbance values obtained after performing MTT assays were normalised relative to vehicle treated control (0.5% DMSO). The final concentration of DMSO in any well was less than 0.5% (v/v) to avoid any contribution of DMSO towards cytotoxicity. Dose response curves were plotted as percentage of vehicle control using GraphPad and the IC_{50} values calculated. Data was appropriately transformed and captured into CalcuSyn software. Using the software, the curves were fitted to linear models using the median effect equation allowing for CI values as a measure of synergy to be simulated at any fa value using the combination index equation. The dose reduction afforded by the different fixed-ratio drug combinations was expressed as the percentage reduction in IC_{50} value of PTX alone.

Table 5.1. Example of a 96 well plate lay out showing the final concentration (in well) of two drugs (PTX and B663) alone as well as two representative FRDC

	B663		Paclitaxel				1:1 (PTX:B663)			1:2 (PTX: B663)		
	1	2	3	4	5	6	7	8	9	10	11	12
A	4 µM	4 µM	0.2 µM	0.2 µM	0.5% DMSO Controls		0.2 µM PTX 0.2 µM B663			0.2 µM PTX 0.4 µM B663		
B	2 µM	2 µM	0.15 µM	0.15 µM			0.15 µM 0.15 µM			0.15 µM 0.3 µM		
C	1.5 µM	1.5 µM	0.1 µM	0.1 µM			0.1 µM 0.1 µM			0.1 µM 0.2 µM		
D	1 µM	1 µM	0.075 µM	0.075 µM			0.075 µM 0.075 µM			0.075 µM 0.15 µM		
E	0.75 µM	0.75 µM	0.05 µM	0.05 µM			0.05 µM 0.05 µM			0.05 µM 0.1 µM		
F	0.5 µM	0.5 µM	0.025 µM	0.025 µM			0.025 µM 0.025 µM			0.025 µM 0.05 µM		
G	0.25 µM	0.25 µM	0.01 µM	0.01 µM			0.01 µM 0.01 µM			0.01 µM 0.02 µM		
H	0.1 µM	0.1 µM	0.005 µM	0.005 µM			0.005 µM 0.005 µM			0.005 µM 0.01 µM		

Experimental design and procedures: HCT-15

In vitro experimentation with HCT-15 cultures were used as a proof of concept prior to initiating expensive animal studies. The requirement to proceed further was that the IC_{50} of the FRDC must be significantly lower than that of PTX alone.

FRDC samples: 1 mg/ml PTX and 2.77 mg/ml B663, i.e. 1.17 mM PTX and 5.85 mM B663 (1:5 molar ratio) were prepared in 100% DMSO. Stock solutions (2 mg/ml) of PTX (2.34 mM) and B663 (4.22 mM) were prepared separately in DMSO and shipped via DHL (in a low temperature “Solution 9” packaging) at 2 - 8˚C to Charles River, Michigan, USA as a pilot to the *in vivo* effiacy studies that were to follow.

The individual drugs were diluted with complete media to make starting stock solutions of 4000 nM and 2000 nM for the B663 and PTX respectively. Solutions were serially diluted 1:3 in complete medium to produce a range of concentrations (0.2 - 4000 nM and 0.1 - 2000 nM respectively). An aliquot of 100 µl of each drug concentration was added to 100 µl of cell suspension thus producing a 1:2 dilution in well to obtain the final testing concentration range.

Similarly, the FRDC was diluted in complete media to make a starting stock solution of 2000 nM PTX and 10000 nM B663 in combination. After consecutive 1:3 serial dilutions, the final concentration range tested in the wells were 0.5 - 1000 nM PTX in combination with 0.25 - 5000 nM B663 thus maintaining the fixed molar ratio of 1:5.

5.3.3. Assessment of additional ABC transporter inhibition

The hypothesis that Riminophenazines inhibit the action of ABC transporters in general was evaluated by Ms. M. Finberg under the supervision of D. Koot. The purpose of such investigations was to establish Riminophenazines as true broad-spectrum resistance circumventers thus increasing the potential scope of the use of this class of drug.

Experimental design and procedures: ASH-3

For preliminary investigation, the intrinsic MRP expressing cell cultures were seeded in 96 well plates (10000 cells/well) and incubated for 24 hrs before the addition of test drug/s.

A checkerboard design was used with a varying final (in well) concentration of doxorubicin (MRP substrate) generally ranging from 0.1 – 2 µg/ml either alone or in combination with three fixed non-toxic concentrations of B663. As such, non-fixed-ratio drug combination experiments were used to assess the chemosensitizing action of Riminophenazines in an *in vitro* MRP expression model.

5.3.4. Neoplastic specificity

Primary human lymphocytes were used in comparison with COLO 320DM cells as an indicator of the cytotoxic specificity for normal versus neoplastic cells allowing for a specificity index to be calculated.

Experimental design and procedures: Lymphocyte isolation and bioassay

Lymphocytes were isolated from healthy human volunteers through the following procedure: 30 ml heparinized blood (0.1 ml heparin / 1 ml blood) was carefully loaded onto 15 ml Histopaque followed by centrifugation at 500 *g* for 25 min. The top plasma player was removed and the lymphocyte /monocyte layer transferred to sterile 50 ml centrifuge tubes; the tubes were filled with RPMI 1640 medium and again centrifuged at 500 *g* for 15min; the supernatant was discarded and the tube again filled with RPMI media before centrifugation at 500 *g* for 10 min; After again discarding the supernatant, the tube was filled with sterile, cold ammonium chloride and placed on ice for 10 min before once more centrifuging at 500 *g* for 10 min, discarding the supernatant, washing the pellet with RPMI and finally re-suspending the cells in 1 ml of RPMI supplemented with 10 % FCS.

The isolated lymphocyte concentration was determined using a haemocytometer and appropriately diluted with full media so as to attain a final seeding concentration of 20000 cells per well. After pre-incubation for a period of 1 hr, 20 µl of various concentrations of each of the drugs (B663, B4125, ETOP, PTX and VIN) were added so as to span the expected dose response range. As was for the COLO 320DM neoplastic cell cultures, 20 µl of an appropriate DMSO percentage was added to all control wells upon which results could be normalized. Cultures were incubated in 5% CO_2 at 37℃ for 3 days before MTT assays were conducted.

5.3.5. Ethical considerations

Research ethics committee approval for the use of primary human lymphocytes was obtained (Project number 164/02 of the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria).

5.4. Results

5.4.1. COLO 320DM neoplastic cell cultures

Dose response curves (Figure 5.1.) were produced for each drug alone as well as for selected fixed molar ratio combinations (10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10) of either B663 or B4125 with ETOP, PTX or VIN. IC_{50} values were estimated from triplicate experiments using Graph Pad software for all of the drugs alone (Table 5.2). As a measure of neoplastic specificity the drugs were also screened against primary human lymphocytes. Specificity (normal/neoplastic) indexes of 1.26, 1.99, 0.62, 1.2 and 4.71 were calculated for B663, B4125, ETOP, PTX and VIN respectively.

CalcuSyn software was used to perform median effect analysis and generate CI index for actual and simulated fa values. The linear correlation coefficient, r, of the respective median effect plots was found to be greater than 0.90 for all drug combinations tested suggesting good fitting to the model.

The extent of drug interaction (CI) was expressed at a nominal fraction affect (fa) level of 0.5 (Table 5.3.). CI values below 1, denoting synergy were found for all tested drug ratios of both Riminophenazines with VIN. Synergy tended to increase as the relative proportion of VIN increased. Concerning Riminophenazine combinations with ETOP, it is apparent that the highest degree of synergy is present at the highest proportion of Riminophenazine and that this positive interaction steadily decreases, finally resulting in antagonism at high ETOP content ratios. A similar dynamic interaction was observed for Riminophenazine combinations with PTX.

The greatest synergy was observed for PTX:B663 (1:10) with an impressive CI of 0.19 which is qualified as strong synergism in the CalcuSyn software manual. This synergy is superior to that obtained for B4125 in spite of B4125 gaining a ~40% improvement in the IC_{50} value. This could be attributed to greater inhibition of Pgp

as result of a mechanism separate to that inducing cell death or could simply be ascribed to the increased hydrophilicity of the TMP derivative.

At this stage of development (and with due consideration to the synergy results attained and the regulatory status of the compounds), the world's best-selling anticancer drug to date - Paclitaxel (PTX), was elected as the lead SC for development in a resistance-circumventing, synergistic combination with the already approved Riminophenazine, Clofazimine (B663).

CI values for PTX:B663 ratios with a greater proportion of B663 were found to be synergistic (CI<1) at all simulated fa levels (0.1 - 0.9). The trend is for synergy to be greater at lower fa levels (Figure 5.2).

This observed synergy is responsible for the large % reduction in the IC_{50} value of PTX (Figure 5.3.). An increasing dose response was observed with increasing B663 levels. The IC_{50} value was reduced 69% from 44.2 nM (for PTX alone) to 13.5 nM when used in a FRDC of 1:5 with B663. This must be taken in the context that the IC_{50} value of B663 is >1000 nM clearly demonstrating that B663 is sensitizing the cultures to the action of PTX through inhibition of efflux pumps and is not necessarily having a direct antiproliferative effect at these concentrations

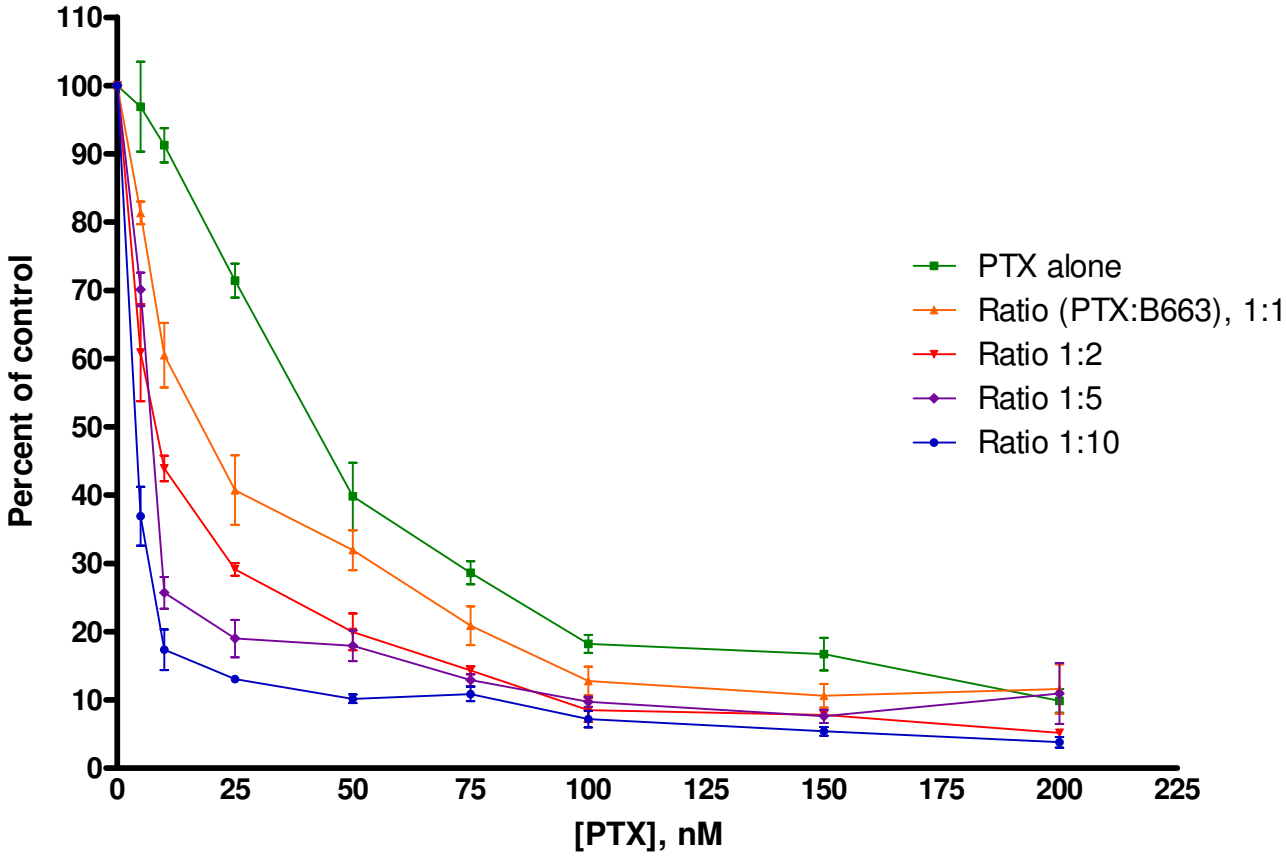

Figure 5.1. The antiproliferative effect of selected fixed ratio drug combinations (FRDC) of PTX and B663 against the COLO320DM cell cultures

Table 5.2. Mean IC_{50} values (nM) of all the tested drugs against COLO neoplastic cell cultures and PHA stimulated primary human lymphocytes

	B663	B4125	ETOP	PTX	VIN
COLO 320DM	1353	800	1370	44	7
Normal Lymphocytes	1700	1594	850	53	33

Table 5.3. Summary of the Combination Index (CI) attained for various fixed ratio drug combinations (FRDC) of Standard Chemotherapeutic (SC) with either of the lead Riminophenazines at a selected simulated *ƒa* of 0.5

CI index values at *ƒa* 0.5						
Molar ratio	**B663**			**B4125**		
(SC: Rimino)	**Etop**	**PTX**	**Vin**	**Etop**	**PTX**	**Vin**
10:1	**1.04**	**1.63**	**0.53**	**1.37**	**1.68**	**0.59**
5:1	**0.94**	**1.32**	**0.72**	**1.37**	**1.19**	**0.53**
2:1	**0.79**	**1.2**	**0.75**	**1.14**	**1.34**	**0.77**
1:1	**0.54**	**0.76**	**0.95**	**0.86**	**2.24**	**0.77**
1:2	**0.54**	**0.43**	**0.89**	**0.56**	**0.75**	**0.91**
1:5	**0.65**	**0.39**	**0.95**	**0.67**	**0.58**	**0.83**
1:10	**0.66**	**0.19**	**0.91**	**0.61**	**0.47**	**0.86**

Synergism (CI<1)

Antagonism (CI<1)

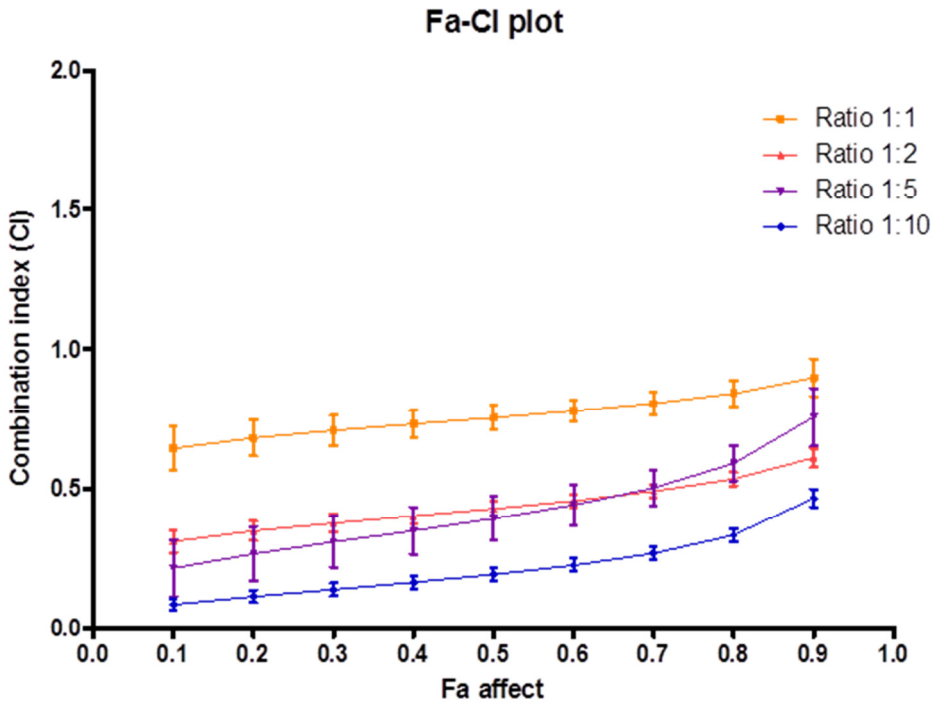

Figure 5.2. Simulated CI index values for selected FRDC of PTX and B663 as a function of the fraction affected (*fa*)

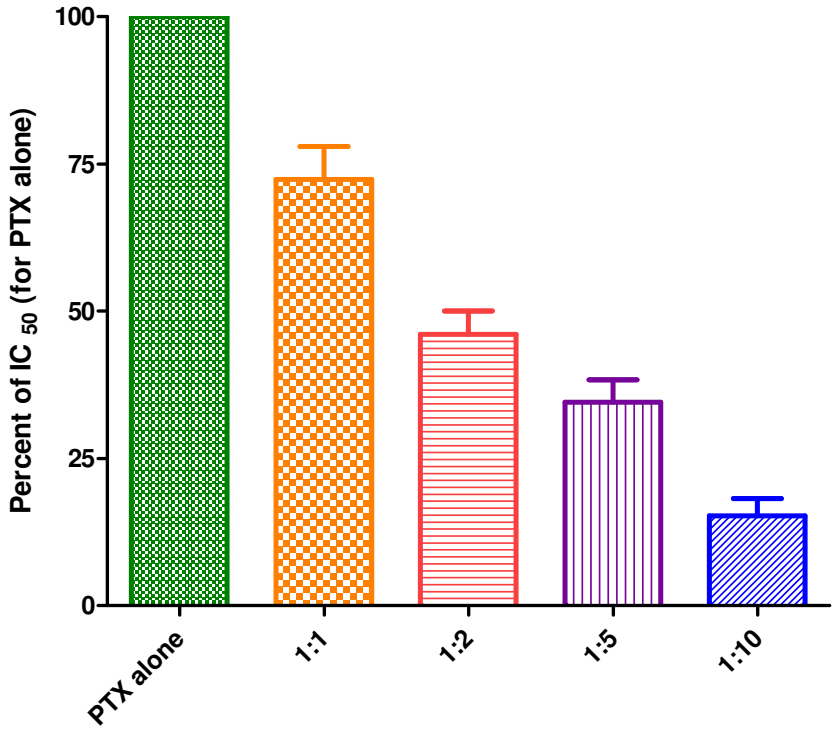

Figure 5.3. Normalised change in the IC_{50} of PTX against COLO 320DM cultures when used in fixed ratio combinations with B663

5.4.2. HCT-15 neoplastic cell cultures

For strategic and practical reasons, *in vitro e*xperiments on the drug resistant HCT-15 neoplastic cell cultures were outsourced to Charles River prior to initiating expensive *in vivo* efficacy studies using implanted HCT-15 cells as a proof that the FRDC does indeed offer antiproliferative benefit. Dose response curves against HCT-15 cells in culture (Figure 5.4.) were produced for PTX alone and for a FRDC of PTX: B663 (1:5) as this was the achieved encapsulation ratio in the NDDS (Chapter 6) used in the *in vivo* study. Similar to the cytotoxicity results observed for COLO 320DM cell cultures, the FRDC showed a large reduction (Figure 5.5) in the dose required to inhibit cell proliferation by 50% (IC_{50}) compared to the cells treated with PTX (alone).

The IC_{50} value was reduced by 72%, from 123 nM for PTX alone to 34.5 nM for the FRDC with B663 (Table 5.4). The IC_{50} of B663 was found to be >2000 nM. This illustrates that HCT-15 cultures are less sensitive (more resistant) than COLO 320DM cells and many other intrinsically resistant tumours (Appendix B). It is evident that a clear chemosensitizing affect is seen at concentrations of B663 that are not directly cytotoxic.

As part of Charles River's standard assay procedure, cisplatin was used as an intra-assay quality control confirming validity of the assay compared to previous experiments. The relatively high IC_{50} value for cisplatin demonstrates the highly resistant nature of these HCT-15 cells for numerous drugs through diverse mechanisms.

Table 5.4. Mean IC_{50} value (nM) of various drugs against HCT-15 neoplastic cell cultures

	B663	PTX	FRDC (PTX:B663, 1:5)	Cisplatin
HCT-15	>2000	123	34.5	10600

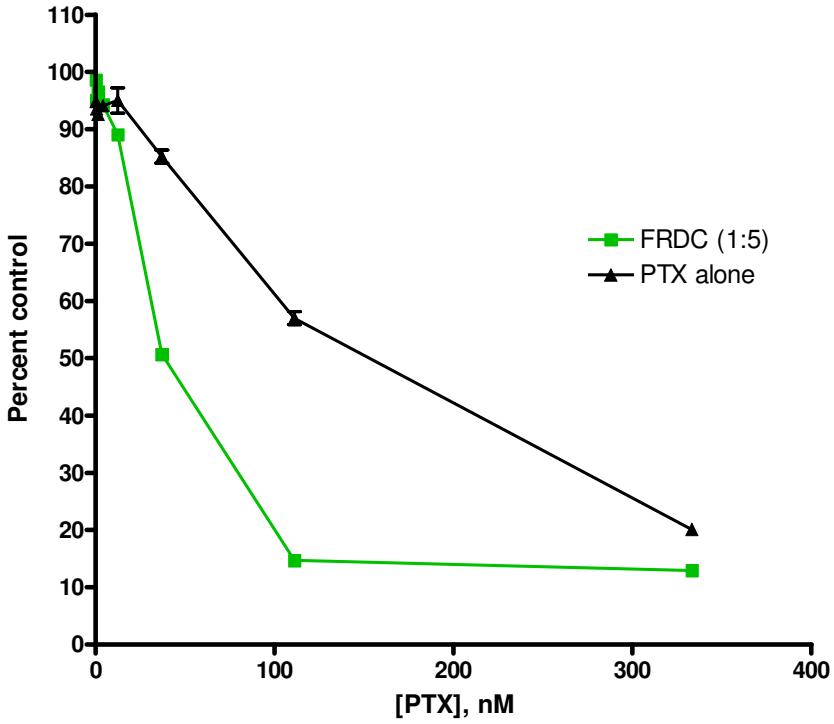

Figure 5.4. Dose response curves for PTX (alone) and the FRDC (PTX:B663, 1:5) against HCT-15 neoplastic cultures

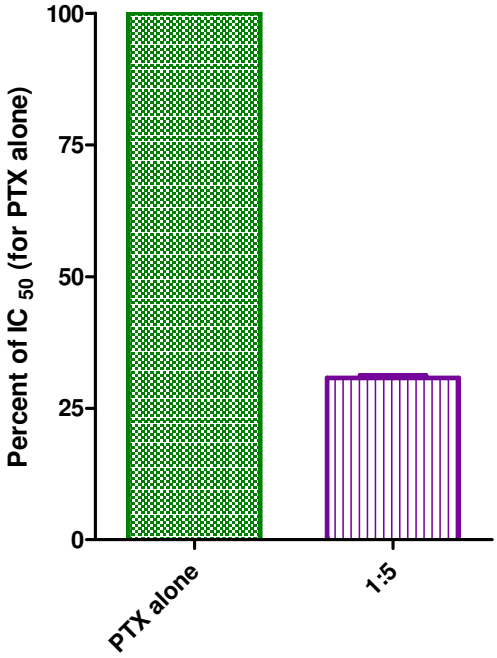

Figure 5.5. Normalised change in IC_{50} value of PTX against HCT-15 cultures when used in a fixed ratio combination with B663

5.4.3. ASH-3 cell line

The MRP expressing ASH-3 cell line was used to assess the ability of three non-cytotoxic concentrations of B663 to potentiate the antiproliferative effect of DOX in a checkerboard manner. The antiproliferative effect of Doxorubicin was shown to increase as the constant dose (non-fixed ratio) of B663 increased (Figure 5.6.). This is strongly indicative that Riminophenazines are inhibiting the efflux action of the MRP pump.

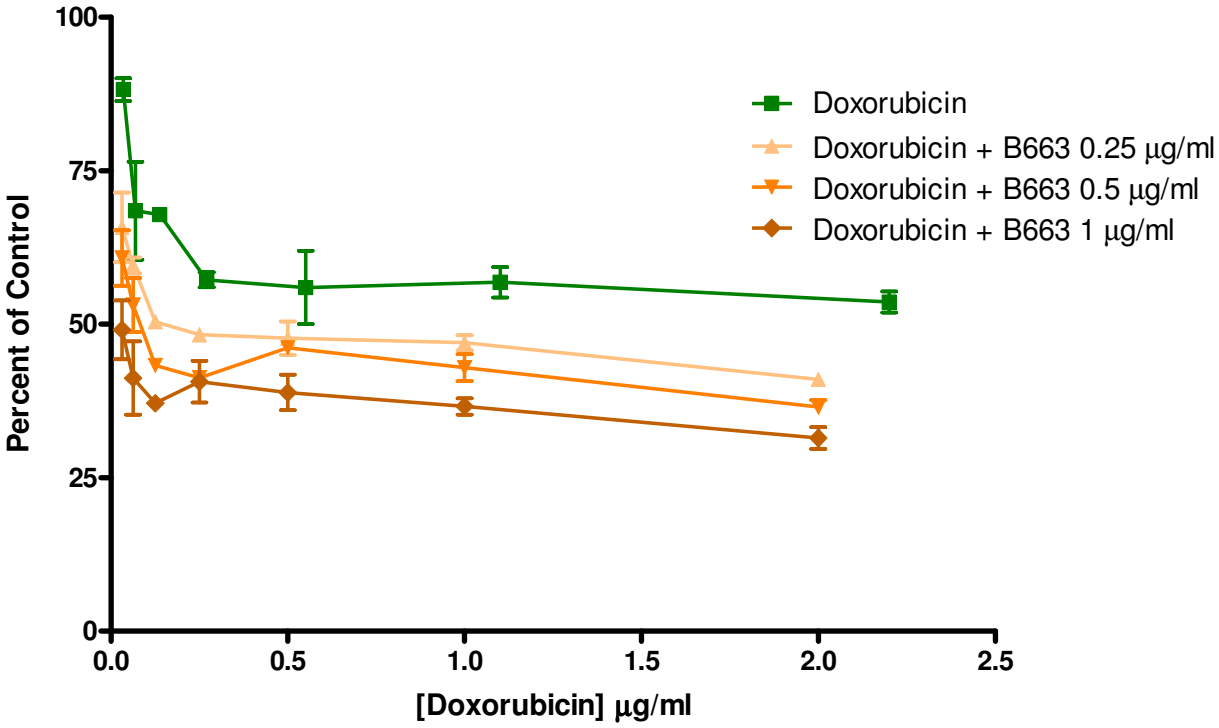

Figure 5.6. The antiproliferative effect of doxorubicin with and without various constant concentrations of B663 against ASH-3 cell cultures

5.5. Discussion and conclusion

For good reason, the evaluation of potential anticancer drugs begins with *in vitro* bioassays prior to pre-clinical animal and finally clinical human studies. This allows the cytotoxicity of various new chemical and biological entities as well as novel combinations thereof to be investigated quickly in a controlled manner at various concentrations against several different cell types and critically assessed thereby justifying the expense of studies that are to follow. *In vitro* bioassays have the advantage of requiring minimal amounts of drug substance and are unaffected by pharmacokinetic effects - this is however, a reductionist point of view and does not consider the effect of the body (absorption, distribution, metabolism and excretion) on the drug/s.

With the advent of programmable liquid handlers, automated high throughput screening (HTS) is facilitated and a huge opportunity exists to screen innumerable drug combinations *in vitro* quickly. This scale of screening is not ethically acceptable, nor rational using *in vivo* models because of the large number of animals required to obtain a statistically valid result and the enormity of possible combinations. [97] Nevertheless, *in vivo* assays employing a whole organism are always required to substantiate findings prior to human trials and to investigate pharmacokinetics, dose ranging as well as to identify organ accumulation and possible adverse effects. Furthermore, the functionality of the dosage form (formulation) needs to be evaluated *in vivo*. Synergistic drug combinations are both ratio and dose level dependent and therefore need to be controlled both spatially and temporally to achieve the best possible interaction.

In this study, for the first time it has been demonstrated that Riminophenazines act synergistically (CI<1) in combination with etoposide, paclitaxel and vinblastine against Pgp expressing COLO 320DM neoplastic cell cultures. It is therefore reasonable to expect that B663 or B4125 could be used (with benefit) within chemotherapeutic regimes involving these Pgp substrates. After considering the potential clinical value, the results of the initial experiments, the physicochemical characteristics and particularly the wide spectrum of use, PTX was selected as the

combination partner for the already registered B663. This combination was further evaluated in an additional neoplastic cell culture and ultimately co-formulated within a NDDS (Stage II) in preparation for *in vivo* investigations (Stage III).

Importantly, synergy affords a reduction in the dose required to produce a particular fractional affect. The elected FRDC of 1:5 (PTX:B663) produced roughly a 70% reduction in the IC_{50} value compared to PTX alone in both of the tested colon carcinoma cell lines, each displaying different levels of drug resistance. This strongly attests to the inhibition of Pgp by B663 increasing the intracellular (active) concentration of PTX making PTX far more efficacious. Over and above the direct antineoplastic effect (at higher Riminophenazine concentrations), there is clearly therapeutic benefit to be gained in terms of PTX dose reduction afforded and therefore reduced drug related adverse effects.

In all three neoplastic cell cultures tested, the synergistic effect was shown to be dose dependent for B663 and observed at concentrations at which little to no direct cytotoxic effect was detectable when used alone. The distinction between potentiation through chemosensitization and true synergy through both drugs possessing a direct effect can however be debated. Regardless of the distinction (which would require a statement of the concentration used), true quantifiable synergistic interactions (defined as greater than additive activity regardless of what model) were attained through the well-established combination index methods of Chou. [94]

Based on IC_{50} values observed for the tested drugs, PTX is roughly 30 fold more cytotoxic than B663 regardless of the cell line used. This implies that a ratio near 1:30 (PTX:B663) would be required before the expected direct cytotoxic action of B663 could be observed over and above the chemosensitizing effect. This has practical bearing in terms of the encapsulation efficacy required within delivery systems, especially when considering the limitations of IV dosing volumes and infusion rates in small animals. This is not to say that these concentrations (of B663) are not clinically achievable particularly if administered independently via the oral route.

Results obtained using the MRP expressing ASH-3 cell line strengthens the case for the use of Riminophenazines as broad-spectrum resistance circumventers and supports their use against MRP and possible all ATP dependent transmembrane efflux pumps. This indirect way of testing drug efflux inhibition should however be supplemented by additional quantitative assays determining the concentration of the an ABC transporter substrate intra- and extracellularly in the presence and absence of Riminophenazines so as to confirm these findings.

Numerous other resistant neoplastic cell types with diverse resistance mechanisms and different sensitivities to Riminophenazines should be investigated in addition to those described in Appendix B. Unfortunately, neoplastic cell cultures with well understood and well reported resistance mechanisms are not readily commercially available. Induction of acquired resistance in-house through clonal selection is laborious and does not necessarily achieve a stable phenotypic (efflux pump) expression. For this reason, future work might do well to explore the use of *ex vivo* patient explants that will truly represent the extent of natural diversity of the disease. Links to personalising a specific FRDC through HTS to a particular patient's cancer phenotype could rationally be pursued. An innovative means whereby this personalised (tailored) approach could be evaluated and the processes optimised is through the use of cancer burdened domesticated animals that will more accurately represent the natural resistance diversity of cancer.