

## 7. *In vivo* models of experimental toxicity and oncology

### 7.1. Introduction

Paclitaxel (PTX), C<sub>47</sub>H<sub>51</sub>NO<sub>14</sub> (molecular weight of 853.9), is a diterpenoid derived from *Taxus brevifolia*. PTX is a potent antineoplastic drug that has demonstrated significant antitumour activity against a wide variety of malignancies particularly ovarian, breast and non-small cell lung cancer. PTX is a cytotoxin active against dividing cells as it is a mitotic inhibitor that promotes the formation of highly stable microtubules that resist depolymerization, preventing cell division and arresting the cell cycle at the G<sub>2</sub>/M phase. [138].

PTX is highly hydrophobic and its intravenous administration is consequently dependent upon a suitable vehicle. Currently PTX is formulated as Taxol® (Bristol-Myers Squibb) within Cremophor EL® (polyethoxylated castor oil) and EtOH (1:1, v/v) to 6 mg/ml. Typically, Taxol will be further diluted with saline to a PTX concentration of 1 mg/ml prior to IV administration. This castor oil based vehicle is thought to be responsible for the hypersensitivity reactions encountered in many patients necessitating pre-treatment with antihistamines (diphenhydramine or cimetidine) and/or a glucocorticoid (dexamethasone). Non target toxicity includes haematological effects (neutropenia), peripheral neuropathy and transient myalgia. [139]

Due to the high occurrence of hypersensitivity reactions and the acquired resistance, many new formulations of paclitaxel are currently in various phases development all around the world. Although many of these formulations (Abraxane, LEP-ETU, Genexol-PM, PGG-PTX, Nanotax and NK105) have successfully eliminated the need for cremophor, they have done little to overcome the problem of Pgp mediated drug resistance. None of these formulations have included a synergistic drug partner to attempt to add cytotoxic potency for a greater range of cancers.

Riminocelles is a sterile co-formulation of an *in vitro* optimized synergistic combination of PTX and B663 prepared in a mixture of DSPE PEG 2000 and lecithin intended to be suitable for parenteral injection. Riminocelles is a nanoparticulate delivery system that has been designed to circumvent resistance and passively accumulate in solid tumour tissue after IV injection.

Prior to first in human clinical trials it is prudent to first evaluate the expected safety and efficacy of the investigational product in animal models in a way that conforms to international best practice and ethics. The contemporary view of pre-clinical toxicity is that one need only determine the specific information deemed pertinent prior to initiating a particular clinical trial. A unique pre-clinical development plan should be devised for each anticancer pharmaceutical considering all available data.

The primary goals of pre-clinical toxicity testing of anticancer drug products are to: identify safe phase 1 clinical trial starting dosages; identify potential organ toxicities and their reversibility; assist in the design of human dosing regimens and escalation schemes. [85, 140] The aim is to gain clinically relevant (predictive) data that can be used to justify (and support) human studies.

Considering the targeting nature (by design) and intent behind the investigational pharmaceutical under development (Riminocelles) - a healthy tumour-free mouse model cannot serve to assess what a true limiting dosage level would be, i.e. a severely toxic dose  $STD_{10}$  (MTD) as recommended by the FDA [84] and ICH [82]. There is no real scientific justification for toxicity testing of cancer-specific therapies in non-tumour-bearing animals. [141]

Accordingly, in an acute toxicokinetic study the goal was not to identify a MTD but rather to obtain important toxicokinetic information regarding the functioning and safety of the drug loaded NDDS. The primary aim of the toxicokinetic study was to assess the true *in vivo* stability of the NDDS and too evaluate the risk of novel toxicities brought about through changes in PTX distribution. As the targeting and efficacy of the NDDS was expected to be an improvement on existing PTX administration protocols, doses of PTX were limited to no higher than currently

used clinical dosages. As such, for acute toxicokinetic studies a single dose of 10 mg/kg PTX as determined to be safe by Gustafson *et al.*, 2005 [142] was used.

Apart from demonstrating safety (in rodent and non-rodent species) through acute and repeat dose toxicity studies as required by international regulatory authorities, a major goal (upon which future studies and financial investment hinge) is to establish the efficacy of the drug product using well-reasoned animal tumour models that are reflective and predictive of the human condition. This provides an opportunity to evaluate different doses and dosing schedules prior to initiating human trials so as to speed the establishment of clinically effective doses in phase I trials.

To ensure safety, it is required that both rodent and non-rodent animals are dosed using a clinically representative regime. Numerous tumour models for the evaluation of drugs have been developed ranging from chemically induced, syngeneic, transgenic and xenografts. Transplantation of human neoplastic cell cultures (xenografts) into immunodeficient athymic (nude) mice is frequently used in pre-clinical studies to evaluate the activity of potential anticancer agents. Subcutaneous implant models are most often employed because of the ease of inoculation and subsequent serial tumour measurement. [143] The use of human xenografts has become the “gold standard” in anticancer drug development and this technique is recommended by regulatory agencies. [144]

Nude mice implanted with the drug resistant HCT-15 colon adenocarcinoma cell line as a model of Pgp mediated MDR cancer, was used to investigate the efficacy and toxicity of two different Riminocelles and Taxol treatment schedules and compared to an untreated control group.

## 7.2. Materials

### Animals

Twelve female BALB/c mice were used for the acute toxicity experiments conducted at the University of Pretoria, Biomedical Research Centre (UPBRC).

Forty female homogenous nude (Crl: Nu-Foxn1<sup>nu</sup>) mice were used in the outsourced Charles River Laboratory (CRL) studies.

### Drugs and formulations

PTX was obtained from Sigma Aldrich (UPBRC) or from Hauser Pharmaceutical Services (CRL) as an off white powder and stored at -20<sup>o</sup>, protected from light.

Taxol® (paclitaxel - 99.8% was obtained from Hauser Pharmaceutical Services as an off white powder and stored at -20<sup>o</sup>, protected from light. Taxol was prepared fresh for each treatment by first dissolving in absolute ethanol, sonicating briefly, adding an equal volume of Cremophor to make a 6 mg/ml solution. Prior to administration it was diluted with saline to produce a clear and colourless 1mg/ml PTX solution.

PTX-Riminocelles™, (PTX [1 mg/ml] and B663 [2.5 mg/ml]) was prepared in the Department of Pharmacology and shipped via DHL (solution 9 packaging) at -20 °C to CRL (Ann Arbor, Michigan, USA) as 40 x 1 ml vials.

## 7.3. Methodology

### 7.3.1. Pilot safety and acute *in vivo* toxicokinetic assessment of Riminocelles

*Experimental procedures and schedule:*

Animals were acclimatized to conditions for a week prior to experimentation. They were individually ear-tagged and fed standard rodent food and water *ad libitum*.

On Day 1 of the pilot safety study the animals were weighed facilitating the calculation of bolus injection volume for each specific mouse:

$$\text{Mouse weight (kg)} \times 10 \text{ mg/kg} / 1 \text{ mg/ml} = X \text{ (ml)}$$

Riminocelles and Taxol were injected IV into the topically anaesthetised tail veins of 6 mice each at a PTX concentration of 10 mg/kg using a 30-gauge needle over ~1 min using a 1 ml Braun syringe. After injection the animals were observed for clinical evidence of toxicity and tolerance.

On day 7, three mice were randomly taken from each of the two groups. Terminal blood samples were drawn under isoflurane anaesthetization, (into heparin blood tubes) by cardiac puncture using pre-heparinised 1 ml needles for toxicity marker profiling. Organs (liver, spleen, kidney, adipose tissue) were collected, weighed and stored at -70°C for possible later drug level quantitation.

On day 8 and 9, the pharmacokinetic study was performed as outlined in Appendix C. Briefly, 5 mice (each) were injected with Riminocelles and Taxol for each time period (30 min, 1, 3, 6 and 24 hr hour). At termination sufficient blood was collected to facilitate both toxicity marker profiling and LC-MS/MS determination (Chapter 8). Five control mice were treated with saline and euthanized after 24 h facilitating comparative toxicity marker profiling and serving as a source of blank matrix samples.

On day 14 the remaining 3 mice in each group were terminated and blood drawn.

Through using this protocol, animal numbers were reduced and plasma toxicity biomarker profiles were attained at 1, 7 and 14 days post administration for both Taxol and Riminocelles.

### **7.3.2. Toxicity marker profiling**

Blood analysis was carried out immediately after collection at the Clinical Pathology Laboratories, Faculty of Veterinary Sciences, University of Pretoria.

The following tests were performed:

Haematological analysis - Haematocrit, haemoglobin concentration, total erythrocyte and leukocyte blood cell counts.

Kidney function markers - Blood urea nitrogen and blood creatinine.

Liver marker enzymes - Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Gamma-glutamyl transpeptidase (GGT).

### **7.3.3. Efficacy assessment**

The purpose of the proposed *in vivo* efficacy studies was to assess the anticancer effectiveness of the novel Riminocelle formulation in comparison to that of Taxol at an equivalent PTX doses using two schedules (Table 7.1.) versus an untreated using a model of Pgp mediated MDR.

Through outsourcing to a GLP facility (Charles River Laboratories) such a study also serves as the required GLP repeat-dose (mimicking the proposed clinical regime) as stipulated by ICH M3R2.

**Table 7.1. Study design**

Group	# Animals	Compound	Route	Schedule	Treatment days	PTX dose (mg/kg/inj.)
1	8	Untreated control	NA	NA	NA	NA
2	8	Taxol	IV	Q7dx4	Day 7, 14, 21, 28	10 mg/kg
3	8	Riminocelles	IV	Q7dx4	Day 7, 14, 15, 28	10 mg/kg
4	8	Taxol	IV	Q1dx7	Day 7-14	10 mg/kg
5	8	Riminocelles	IV	Q1dx7	Day 7-14	10 mg/kg

## Procedures

### *Mice and Husbandry*

Female mice (Crl:NU-Foxn1nu) of age 8-9 weeks were obtained from Charles River Laboratories. The mice were allowed to acclimate for 5 days. The mice were fed standard irradiated rodent food and water *ad libitum*. The mice were housed in static cages with sterile bedding inside clean rooms that provide HEPA filtered air into the barrier environment at 100 complete air changes per hour. The environment was controlled to a temperature range of 21 ± 2°C and a humidity range of 30-70%. All treatments, body weight determinations, and tumour measurements were carried out in these sterile conditions.

All mice were observed for clinical signs at least once daily. Mice with tumours in excess of 1000mg or with ulcerated tumours were euthanized, as were those found in obvious distress or in a moribund condition.

### *Cell Preparation and implantation*

HCT-15 human colon adenocarcinoma cells were cultured using RPMI 1640 supplemented with 10% non-heat inactivated fetal bovine serum, 1% (1M HEPES), 1% sodium pyruvate, in 5% CO<sub>2</sub> and 95% air humidified atmosphere at 37 °C. The HCT-15 cells were detached from the flasks using 0.25% trypsin/2.21mM EDTA in HBSS per each flask. The HCT-15 cells were collected through centrifugation at 300 *g* for 8 minutes at 4 °C. The cell pellets were suspending in complete media. The viability of the HCT-15 cell suspension was determined using trypan blue exclusion with a haemocytometer both before and after the inoculation period. Prior to implantation the cells were resuspended in 50% serum-free RPMI media and 50% Matrigel® to obtain a cell concentration of 5x10<sup>6</sup> cells/200 µl injection per mouse.

Test mice were implanted subcutaneously high in the right axilla (just under arm) on Day 0 using a 27-gauge needle and a 1 ml syringe. The cell suspension was maintained on wet ice to minimize loss of viability and inverted frequently to maintain a uniform cell suspension during the inoculation procedure.

### *Treatment*

Considering that a subcutaneous tumour is only definitely palpable at >100 mg. [145] Treatment was to begin once the tumours reach 100-200 mg (target 150 mg). [146] Animals were weight matched and assigned to respective groups such that the mean tumour burden in each group was within 10% of the overall mean. Before dosing, each animal was weighed and an appropriate dose volume of the respective [1 mg/ml] PTX formulations (Riminocelles and Taxol) was calculated via the following formulae: Mouse weight (kg) x 10 (mg/kg) / 1 mg/ml = X (ml). Slow injections into the tail vein typically lasted 1min. Appropriate precautions for normal IV chemotherapeutic administration were taken.

### *Tumour measurements and Efficacy endpoints*

Tumour measurements were recorded three times weekly. Tumour burden (mg) was estimated from calliper measurements by the formula for the volume of a prolate ellipsoid assuming unit density as [147, 148]:

$$\text{Tumour volume (mm}^3\text{)} = (\text{width}^2 \times \text{length (mm)}) \times 0.5$$

(Specific gravity assumed to be 1 g/cm<sup>3</sup> therefore mm<sup>3</sup> = mg)

Tumour Growth Delay (T - C value) was used to quantify efficacy where C is the median time in days required for a particular tumour to reach a predetermined mass and T is the median time required for the treatment group to reach the same predetermined evaluation size. Tumour Growth Delay was measured at a tumour weight of 750 mg.

Tumour-Cell-Kill was used as a secondary efficacy endpoint. The log<sub>10</sub> Tumour-Cell-Kill was calculated from the formula:

$$\text{Log}_{10} \text{ tumour cell kill total (gross)} = [\text{T} - \text{C value in days} / 3.32 (T_d)]$$

Where  $T_d$  is the tumour-volume doubling time (in days) estimated from the least squares best-fit straight line from a log-linear growth plot of the control group whilst in exponential growth, 200-800 mg range. [146, 147] The value 3.32 is derived from the number of doublings required for a cell population to increase 1 log<sub>10</sub> unit. [148]

For a treatment duration of between 5 and 20 days a Gross Log<sub>10</sub> tumour cell kill of >0.7 is required before a treatment is considered as active and a value of >2 would be required to produce tumour regressions in most models. [149]

Tumour growth inhibition values (%T/C) were determined every second day through comparison of the median tumour burden (mass) for each group relative to the untreated control. The Drug Evaluation Branch of the Division of Cancer Treatment, National Cancer Institute (NCI) considers a T/C ≤ 42% as significant

antitumour activity. A value <10% is considered highly active and justifies a clinical trial. [149]

#### *Assessment of Side Effects (GLP repeat dose toxicity assessment)*

Mouse body weights were recorded three times weekly. Treatment-related weight loss in excess of 20% was considered unacceptable and the animals were to have been euthanized. A dosage level was considered as tolerated if treatment-related weight loss (during and two weeks after treatment) is <20% and mortality during this period in the absence of potentially lethal tumour burdens is  $\leq 10\%$ . All mice were observed for nadir weight loss and weight loss return.

Upon death or euthanasia, all mice underwent necropsy to provide a general assessment of potential cause of death and perhaps identify target organs of toxicity. The presence or absence of any tumour metastases was also noted.

#### **7.3.4. Ethical considerations and committee approval**

All studies complied with the ethical approval given for project number H10-09 (University of Pretoria, AUCC) - Appendix A.

All animal studies were designed following the philosophy of the 3 R's (Reduce, Refine, Replace). Humane endpoints as stipulated by the SA National Standards (SANS 10386:2008) were followed. The veterinarian in charge had full authority to terminate any animal or the entire study in the event that any of the animals were deemed to be suffering.

All procedures carried out abroad were conducted in compliance with all the laws, regulations and guidelines of the National Institutes of Health (NIH) and with the approval of Discovery and Imaging Services, Ann Arbor's (DIS-AA) Animal Care and Use Committee.

### **7.3.5. Statistical analysis**

Appropriate statistical comparisons were performed to determine whether the difference between various groups was statistically significant. A  $P < 0.05$  was considered significant.

Concerning the efficacy assessment, the median times to evaluation size (750mg) for all study groups were first analysed by application of the log rank (Kaplan-Meier) test to determine if any significant differences existed between groups. Upon identification that significant differences existed, multiple comparisons were performed to identify the groups that differ from one another.

One-way ANOVA was used to compare the weight differences of the various treatment and control group. For specific time points, Dunnett's multiple comparison (with control) post tests were used to identify statistically significant treatment-related weight loss.

Statistical significance was determined using Microsoft Excel analysis tools and GraphPad (Version 5).

## **7.4. Results**

### **7.4.1. Acute toxicokinetics**

All injections were well tolerated during the first 5 minutes post administration and subsequent observation points. Furthermore, the Riminocelle formulation was locally well tolerated at the injection site.

The animals in each group were monitored 3 times a day and weighed every second day for latent effects up to 14 days (Figure 7.1.). There is no statistically significant difference between the comparative weights on day 0 and day 14 for each respective treatment group. A slight trend can be seen in the second week for the Riminocelles group to increase in weight and the Taxol group to lose weight.

Poor weight matching and triaging into groups at the start of study could explain the observed difference in weight-time profiles between Riminocelles and Taxol. No clinical signs of toxicity were evident throughout the observation period.

Terminal blood samples drawn from 3 mice after 24 h in the pharmacokinetic study and from 3 mice after 7 days and 14 days post administration were assessed for plasma markers of toxicity and found to be within the normal range (Table 7.2.). The presence of GGT in the plasma of the Taxol treated group is noteworthy. On occasion, insufficient blood was collected to facilitate replicate measures of certain markers.

All carcasses within this initial pilot study underwent gross necropsy examination. No obvious lesions could be identified in either of the treatment groups. Macroscopic evaluation could not confirm specific or consistent organ pathology and therefore histopathology was not deemed prudent. Of mention is the yellow discoloration of body fat that is prominent even 14 days after acute administration of Riminocelles.

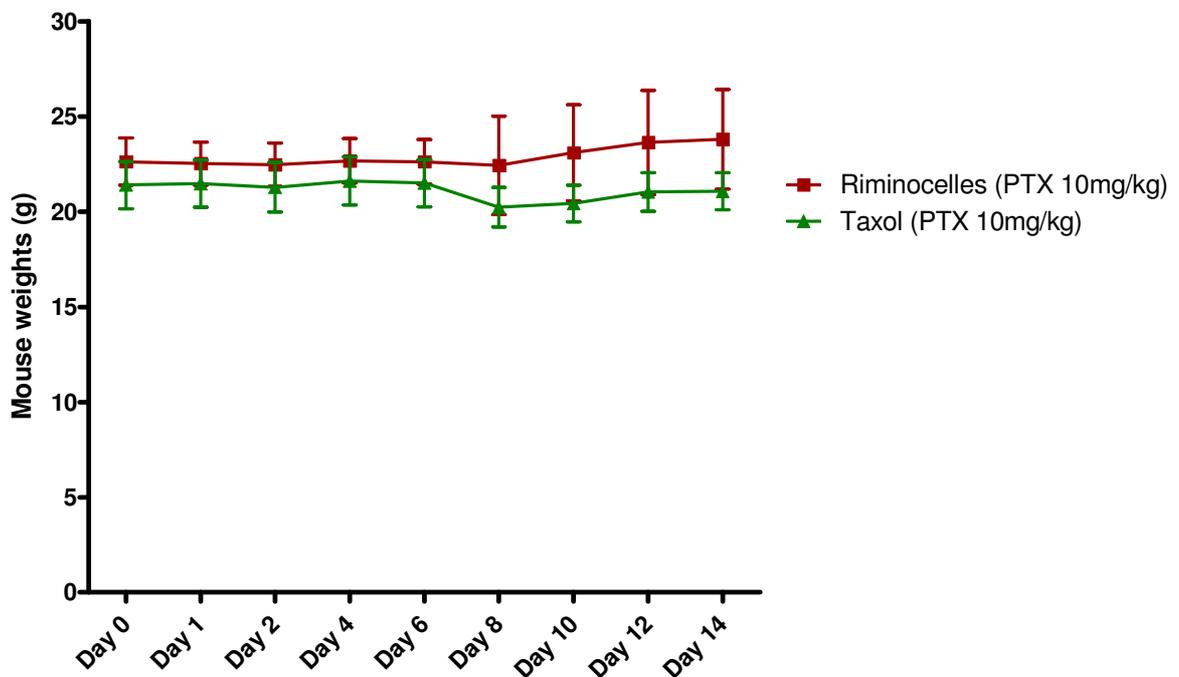


Figure 7.1. Weight changes after acute IV injection of Riminocelles and Taxol

**Table 7.2. Plasma toxicity biomarkers 1, 7 and 14 days after acute IV injection of Riminocelles and Taxol at a PTX dose of 1 mg/ml (n = 3)**

	Riminocelles						Taxol					
	Day 1		Day 7		Day 14		Day 1		Day 7		Day 14	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<b>Clinical chemistry</b>												
ALT (IU/l)	31.2	5.1	26.3	2.5	22.0	*	28.6	2.7	33.3	18.2	26.7	6.4
AST (IU/l)	89.2	10.8	92.7	28.7	71.0	*	113.4	32.0	127.7	16.4	97.3	12.1
GGT (IU/l)	0.0	0.0	0.0	0.0	0.0	*	2.2	2.2	3.0	2.0	3.3	2.5
Urea (mM)	6.2	1.0	4.6	0.3	5.9	*	8.0	1.1	5.8	1.3	5.8	0.8
Creatinine (µM)	<18	0.0	<18	0.0	<18	*	<18	0.0	<18	0.0	<18	0.0
<b>Haematology</b>												
Hb (g/l)	135.8	3.3	148.5	3.5	134.0	18.4	138.8	4.7	131.3	10.6	135.3	3.8
RCC (x10 <sup>12</sup> /l)	8.6	0.3	9.8	0.2	8.5	1.1	8.8	0.3	8.7	0.4	8.4	0.4
HT (l/l)	0.4	0.0	0.5	0.0	0.4	0.1	0.4	0.0	0.4	0.0	0.4	0.0
MCV (fl)	48.0	1.1	46.8	0.4	46.5	1.3	47.0	0.8	47.2	4.2	48.2	1.7
MCH (g/dl)	15.7	0.3	15.2	0.0	15.8	0.0	15.7	0.1	15.1	1.6	16.1	0.6
MCHC (g/dl)	32.8	0.2	32.5	0.2	34.0	0.8	33.3	0.6	32.0	0.7	33.4	0.4
WCC(x10 <sup>9</sup> /l)	5.3	0.5	7.1	2.1	6.0	0.4	4.1	1.1	9.4	5.1	5.2	0.5
Neutrophils (x10 <sup>9</sup> /l)	1.4	0.3	1.9	0.2	1.5	0.9	1.0	0.6	2.9	3.6	1.2	0.3
Lymphocytes (x10 <sup>9</sup> /l)	3.7	0.4	4.9	1.7	4.2	0.3	2.9	1.5	5.2	1.6	3.7	0.3
Monocytes (x10 <sup>9</sup> /l)	0.1	0.1	0.3	0.1	0.2	0.1	0.1	0.1	1.0	1.2	0.3	0.2
Eosinophils (x10 <sup>9</sup> /l)	0.1	0.2	0.0	0.1	0.0	0.0	0.1	0.1	0.2	0.0	0.1	0.1
Basophils (x10 <sup>9</sup> /l)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0

\* Due to the low volume of blood drawn, replicate measure could not be attained

#### **7.4.2. GLP repeat dose toxicity**

A GLP repeat dose toxicity assessment of Riminocelles and Taxol was performed as part of the efficacy study. An equivalent PTX dose of 10 mg/kg for Riminocelles and Taxol was evaluated using two proposed dosing schedules of once a day (repeat for 7 days or unacceptable toxicity) and once a week repeat until progression or toxicity. Weight changes in tumour bearing mice were observed as an indicator of drug toxicity every second day until the end of the study (Figure 7.2.). Percent body weight loss at nadir and day of nadir were recorded (Table 7.3.).

Treatment began on day 7 when the mean tumour burden was 160 mg (range, 151-168 mg). The mean group body weight was well matched at the initiation of therapy (range, 22.5-23.2 g).

Statistical analysis of the percent body weight changes on day 14 revealed a statistically significant difference ( $P < 0.05$ ) between the control group and the Taxol (QDx7) group using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post-test (Figure 7.3.). Regardless, as these weight changes were minimal  $< 20\%$ , at this dosage both treatment regimens are considered well tolerated.

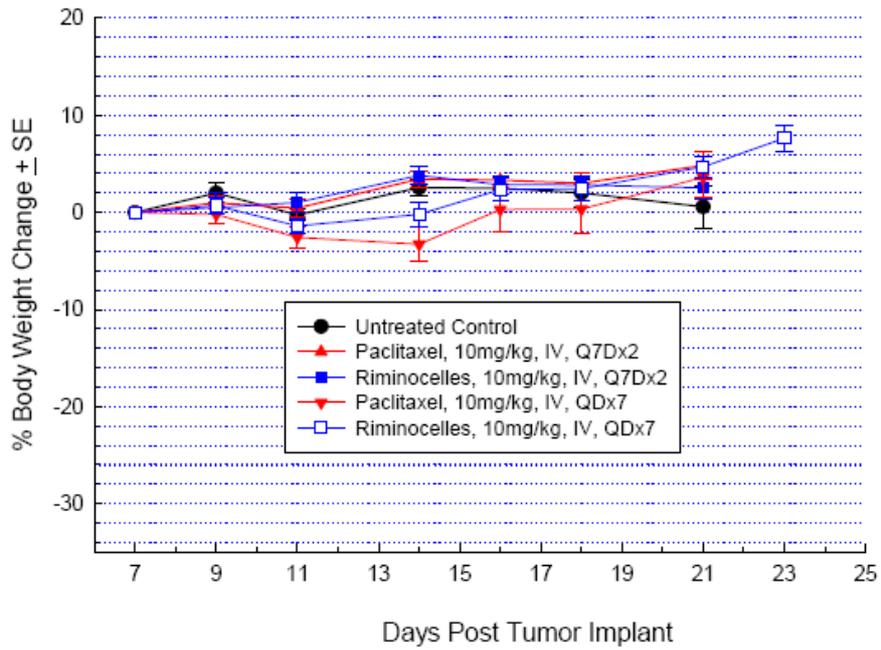


Figure 7.2. Percent body weight change for the respective groups for the duration of the study. Treatment began on day 7

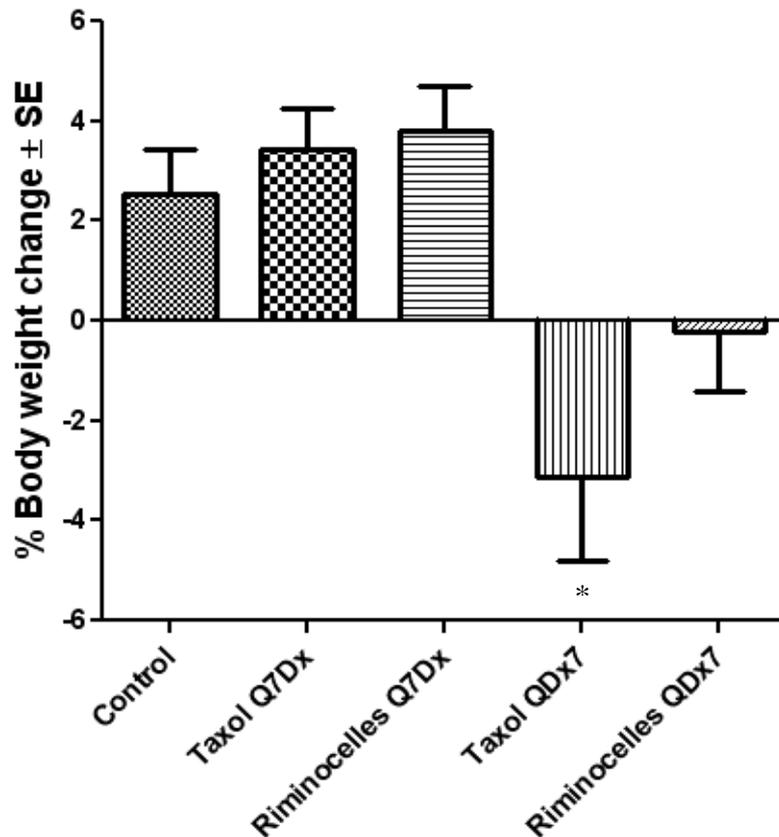


Figure 7.3. Percent body weight change on day 14 after dosing tumour bearing mice with Riminocelles and Taxol (10 mg/kg PTX). n = 8. One way ANOVA with Dunnett's multiple comparison post-test. \* P<0.05

### 7.4.3. Efficacy assessment

The control group reached a tumour evaluation size (750 mg) after 16 days and were all terminated due to tumour burden >1 g on day 22.

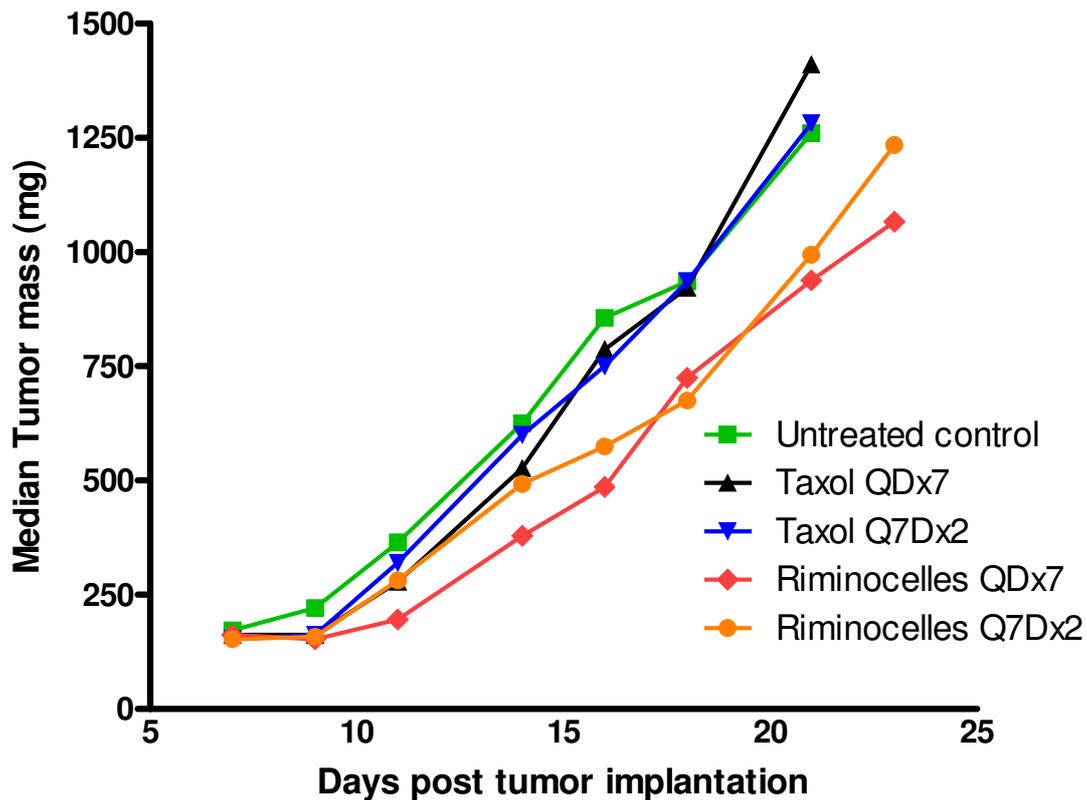
The activity of two different schedules of Taxol and Riminocelles against HCT-15 human xenografts is represented by the median tumour burden over time (Figure 7.4.), as this is the manner prescribed by the NCI for tumour-mass information. [149] It is immediately apparent that Riminocelles out performed Taxol. The log rank statistic for the survival curves was greater than would be expected by chance, that is to say there is a statically significant difference between the curves with a P value <0.001. A multiple comparison post-test was used to isolate the groups that differ from others. Treatment with Riminocelles at 10mg/kg following a schedule of QDx7 or Q7Dx4 produced statistically significant growth delays (T-C) of 3.2 and 2.7 days respectively at an evaluation size of 750 mg whilst treatment with Taxol did not result in any significant tumour growth delay (Table 7.3.). The tumour volume doubling time ( $T_d$ ) was determined to be 4.2 days and is within the historical range of the model. Tumour cell log kill (gross) is reported in Table 7.3. Treatment with Riminocelles following a schedule of QDx7 achieved the greatest tumour cell log kill value of 0.23.

Percent T/C values are shown in Figure 7.5. from the day treatment started to day 22 when the mice in the control group were terminated. The best %T/C was 54% produced by Riminocelles following a daily schedule after 4 days of treatment (day 11).

Previous calculations have considered only the median tumour burden of the groups. In Figure 7.6. the fate of individual mice is considered. In the Riminocelles group following a daily treatment schedule a greater proportion of the mice survive for longer. Treatment with Taxol was observed not to increase the time for tumour burden to reach >1 g.

Treatment with Riminocelles was well tolerated when administered IV. There were no clinical signs of toxicity or effect on vital organ function and necropsy findings

were limited to what appeared to be mild jaundice and yellow staining of the abdominal fat (Figure 7.7 and 7.8.) that can be attributed to riminophenazine disposition.



**Figure 7.4. Median tumour burden-time profile of HCT-15 subcutaneous xenografted human tumours in nude mice. Mice were treated with 10 mg/kg PTX as either Riminocelles or Taxol following two different schedules. Tumour growth delay measurements were determined at a size of 750 mg**

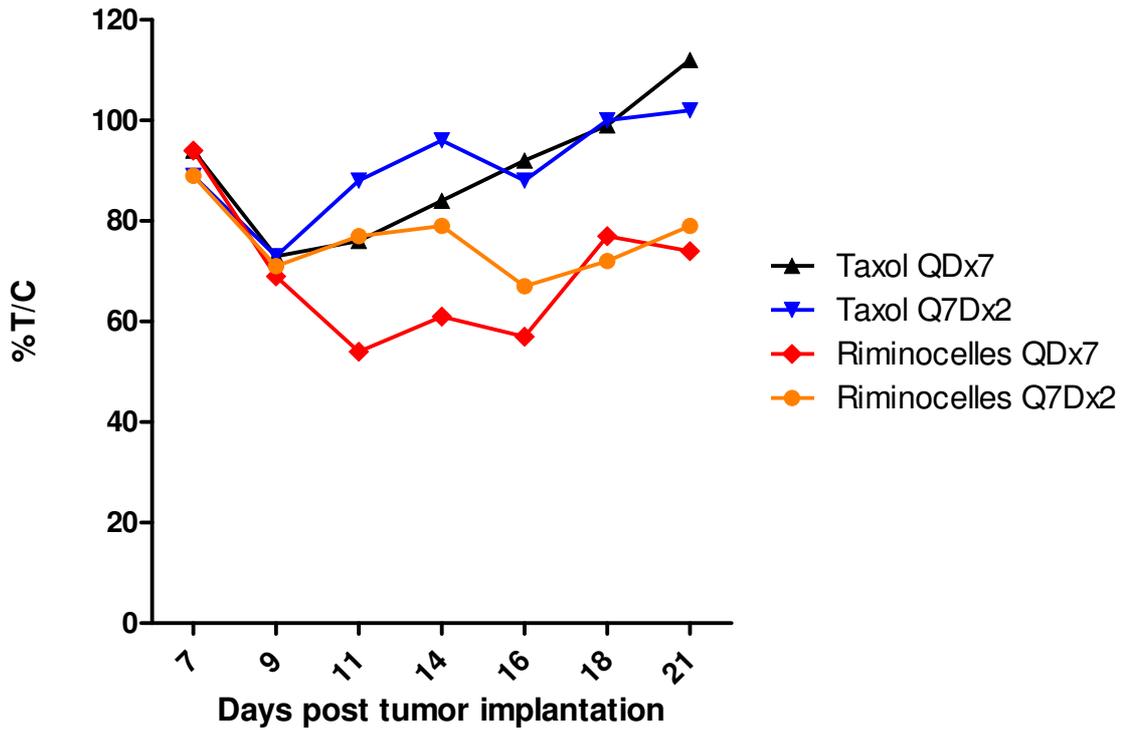


Figure 7.5. Tumour growth inhibition relative to untreated control over time expressed as % T/C

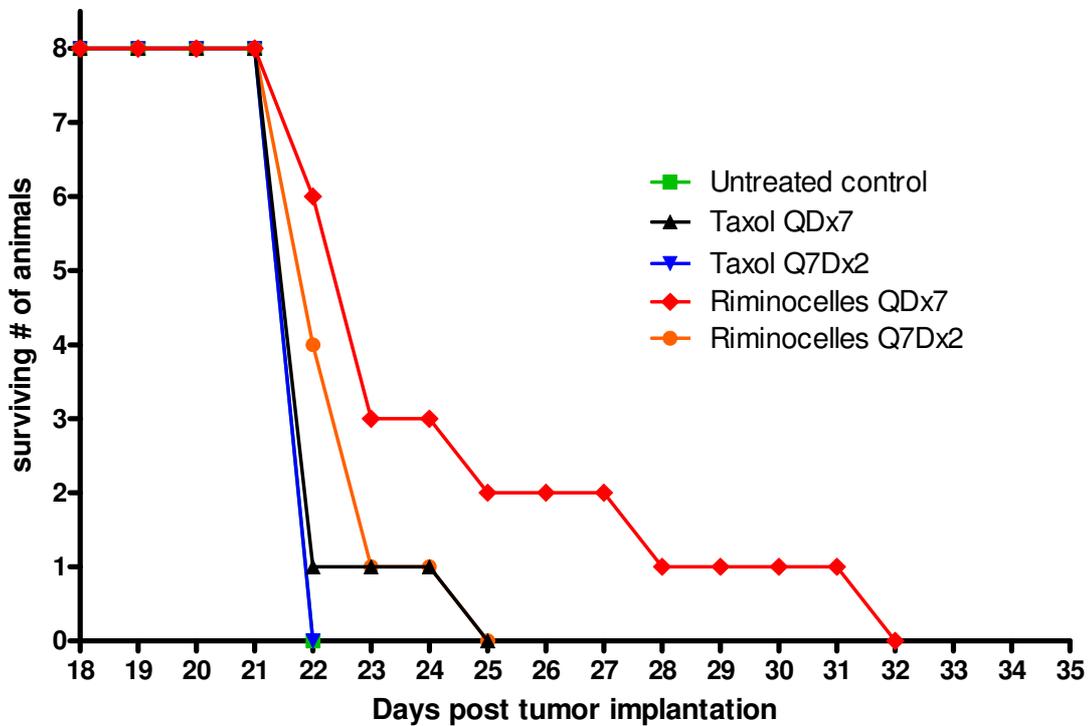


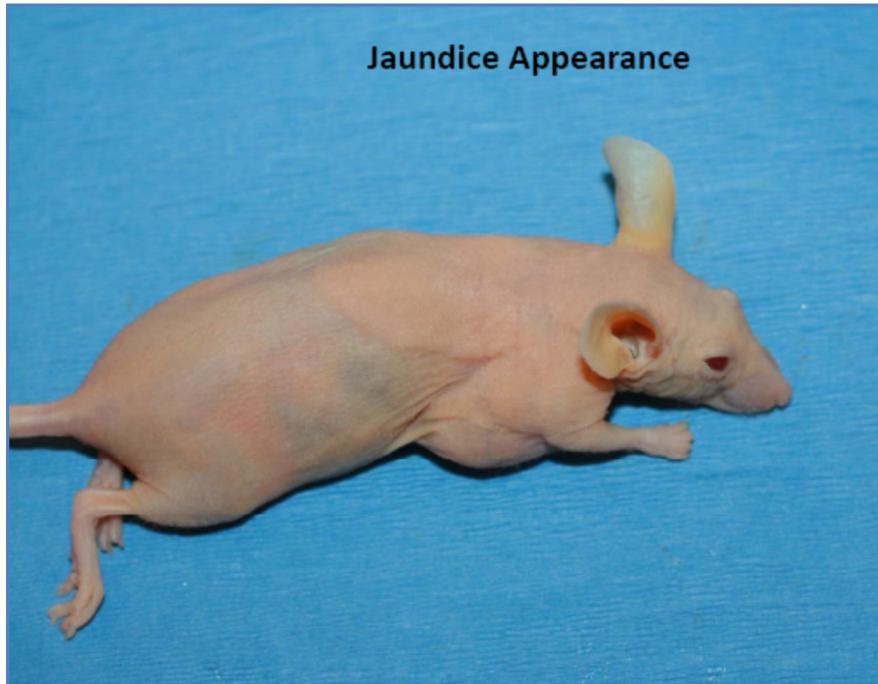
Figure 7.6. Time to tumour burden of 1000 mg for individual mice. The survival of Taxol treated mice (Q7Dx2) was no different to the untreated control

Table 7.3. Summary: Safety and efficacy of Riminocelles in comparison with Taxol

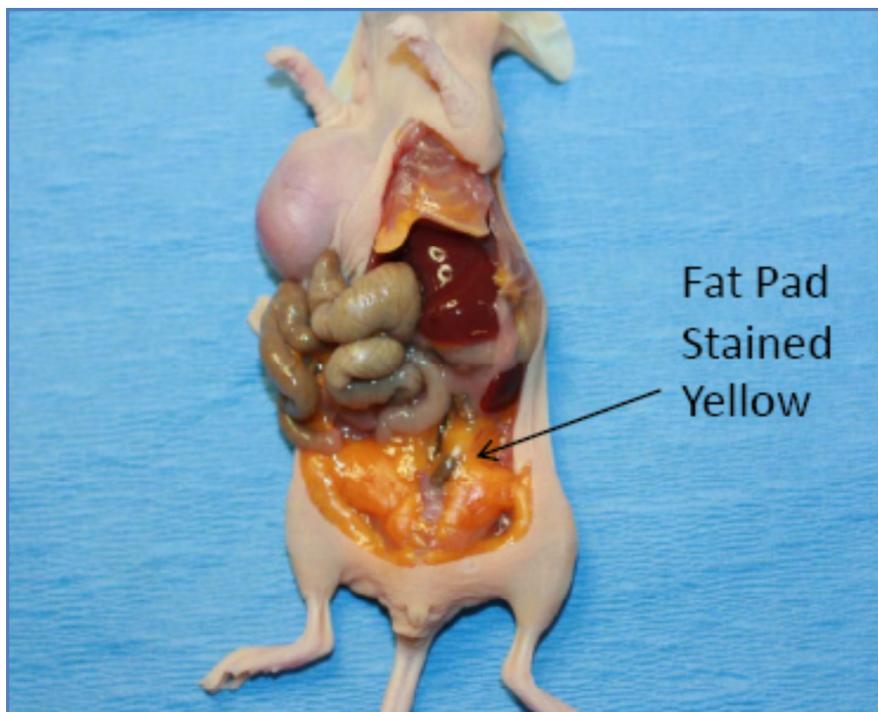
Treatment	Dose <sup>a</sup> (PTX) <sup>b</sup> (B663)	Schedule	Route	% Body wt. loss at nadir (day of nadir)	Rx related (day of nadir)	Tumour growth delay (day of nadir)	Tumour cell log kill (day of nadir)
<b>Control</b>	10 mg/kg <sup>a</sup>	NA	IV	0.4 (11)	0/8	NA	NA
<b>Taxol</b>	10 mg/kg <sup>a</sup>	QDx7	IV	3.5* (14)	0/8	0.5	0.04
<b>Taxol</b>	10 mg/kg <sup>a</sup>	Q7Dx2	IV	+	0/8	0.6	0.04
<b>Riminocelles</b>	10 mg/kg <sup>a</sup>	QDx7	IV	1.3 (11)	0/8	3.2*	0.23
<b>Riminocelles</b>	25 mg/kg <sup>b</sup>						
<b>Riminocelles</b>	10 mg/kg <sup>a</sup>	Q7Dx2	IV	+	0/8	2.7*	0.19
	25 mg/kg <sup>b</sup>						

+ Weight increases

\* P<0.05



**Figure 7.7. Mild “jaundice appearance” that occurred after Riminocelle administration reminiscent of characteristic clofazimine skin discoloration**



**Figure 7.8. Necropsy revealed yellow/orange stained fat tissue due to clofazimine disposition after Riminocelle administration**

## 7.5. Discussion and conclusion

The practicality of conducting pre-clinical studies using animal models must be weighed against what specific research questions have been raised and the information that can be gained. Ultimately, the value of any model lies in its ability to be predictive of the human scenario.

After demonstrating the IV suitability, local tolerability and lack of toxicity produced by Riminocelles in acute toxicity studies using normal healthy mice, the efficacy of Riminocelles was compared to Taxol at an equivalent PTX concentration of 10 mg/kg using highly drug resistant HCT-15 human colon adenocarcinoma cell cultures xenografted subcutaneously in nude mice. This study was performed as a proof of concept using a cancer cell model that demonstrates Pgp mediated MDR.

This study was designed so as to directly compare Riminocelles to that of the commercial formulation, Taxol. Crudely, the effect shown compared to Taxol can then be used as a reference to allow comparison with numerous other re-formulations that have already been or remain to be developed and evaluated *in vivo* with a parallel Taxol treatment group.

In terms of efficacy endpoints: the tumour cell kill values and the % T/C values attained for Riminocelles (while far superior to that attained for Taxol) are below that advocated by the NCI as indicating substantial activity warranting the initiation of clinical trials. Although Riminocelles statistically ( $P < 0.05$ ) outperformed Taxol in terms of efficacy, in this particular model of intrinsic Pgp expression, a PTX dose of 10 mg/kg was not sufficiently potent and the colon adenocarcinoma tumours still progressed rapidly.

Time to a maximum tumour burden of 1 g for individual mice promisingly revealed more mice in the Riminocelles treatment group to survive for longer compared to the Taxol treated groups. One mouse in particular (representing 12.5% of the population) following a daily treatment schedule survived for an additional 7 days compared to the Taxol group again highlighting the superiority of the novel co-

formulation. A shortcoming of this study was that it was not powered to distinguish whether the advantage gained is due to the fixed ratio drug combination or as result of the nanoparticulate delivery vehicle. To this end, future experimental designs could foreseeable include an additional group where clofazimine is administered via oral gavage in addition to Taxol.

In addition, future studies should make use of cancer types for which PTX is clinically indicated such as ovarian, breast and lung xenografts. The ICH S9 guideline [82] clearly stipulates that it is not a requirement to use the proposed clinical cancer type in pre-clinical studies. In clinical trials, all unresectable and refractory cancer patients could be potential volunteers.

In terms of safety: As prescribed by ICH M3R2, Riminocelles has been tested in a GLP compliant repeat dose toxicity study using a clinically representative dosage form. The lack of significant weight changes and the absence of acute toxicity biomarkers indicate that the dose of Riminocelles can be substantially increased (contrary to Taxol that incurred significant weight loss when administered daily). Future pre-clinical efficacy studies should be aimed at investigating a range of doses so as to determine the MTD of the co-formulation remembering that a tumour model is required to accurately assess the toxicity of cytotoxic drug products that by design are meant to distribute specifically to solid tumour tissue.

As the oral LD<sub>50</sub> for B663 in normal mice is >4 g/kg [31] it is reasonable to assume that the dose of B663 could be increased several fold without incurring toxicity. The current encapsulation efficacies achieved within Riminocelles would seemingly limit the B663 dose considering the maximum acute IV dosing volume that can be given safely to a mouse (<400 µl). Should volume restrictions and encapsulation efficacy impede dose increase, oral supplementation with B663 could be considered. The discoloration of adipose tissue and skin is an expected and reversible feature of B663 administration and should not be misinterpreted as jaundice nor as a dose limiting adverse effect. Specific distribution to and retention by fat tissue of B663 for sustained periods is a clear indicator that specific targeting to the tumour (in the most part) has not been achieved.

Before suggesting an appropriate starting dose for first in man studies, all the available pre-clinical data, particularly the pharmacokinetic data should be considered (chapter 8). Currently Taxol is being used clinically at several dosages and schedules with the optimal regimen not yet being clear. [138] The most common regimens (following appropriate premedication) are IV administration of 135 or 175 mg/m<sup>2</sup> over 3-24 h every three weeks. Shorter dosing intervals have typically been met with greater adverse effects presumable due to the cremophor vehicle. [135] It this study it has been demonstrated *in vivo* that novel cremophor-free vehicles could conceivable allow for a re-evaluation of the optimal PTX dosing schedule allowing for a greater cumulative dose to be administered in a shorter period of time (once daily for a week, or longer - if tolerated).

A repeat dose toxicity study in a second species (non-rodent) animal model is required prior to initiating clinical studies, as stipulated by ICH M3R2. [88] Such a repeat dose study will allow for a clinical study of the same duration to be initiated. Should a measurable benefit be seen in human cancer patients there would be no justification to stop treatment. [88] Strategically, such a repeat dose study in non-rodent animals mimicking the proposed clinical schedule is to be conducted last, only after rodent efficacy, toxicity and pharmacokinetic data has been scrutinised and progression justified.

In summation, the Riminocelles system was shown to be safe and efficacious in a human xenograft model of Pgp mediated drug resistant cancer cells. Riminocelles exhibits less toxicity and greater efficacy than Taxol. This study serves as a proof of concept, demonstrating that B663 combination with PTX is rational and can be used with benefit against Pgp expressing cancers.

This study is in support of previous clinical studies evaluating the anticancer activity of B663 [33, 34] The value of including B663 (even if administered orally as Lamprene) within taxane and additional combination chemotherapeutic regimens (doxorubicin) is definitive and should be considered by clinicians as a salvage route when faced with refractory cancer.