

## **Anticancer efficacy and toxicokinetics of a novel paclitaxel-clofazimine nanoparticulate co-formulation**

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### **Keywords**

Paclitaxel; Clofazimine; DSPE-PEG 2000, Lipopolymeric micelle; Toxicokinetics; Efficacy

### **Abbreviations**

MDR, Multi-drug resistance; FRDC, Fixed-ratio drug combination; NDDS, Nanoparticulate drug delivery system; Paclitaxel, PTX; Clofazimine, B663

## Abstract

Contemporary chemotherapy is limited by disseminated, resistant cancer. Targeting, nanoparticulate drug delivery systems that encapsulate synergistic drug combinations are a rational means to increase the therapeutic index of chemotherapeutics. A lipopolymeric micelle co-encapsulating an *in vitro* optimized, synergistic fixed-ratio combination of paclitaxel (PTX) and clofazimine (B663) has been developed called Riminocelles™. The present pre-clinical study investigated the acute toxicity, systemic exposure, repeat dose toxicity and efficacy of Riminocelles in parallel to Taxol® at an equivalent PTX dose of 10 mg/kg. Daily and weekly dosing schedules were evaluated against Pgp expressing, human colon adenocarcinoma (HCT-15) xenografts implanted subcutaneously in athymic mice. Riminocelles produced statistically significant ( $p < 0.05$ ) tumor growth delays of 3.2 and 2.7 days for the respective schedules in contrast to Taxol delaying growth by 0.5 and 0.6 days. Using the control tumor doubling time of 4.2 days, tumor-cell-kill values of 0.23 for Riminocelles and 0.04 for Taxol following daily schedules were calculated. A significant weight loss of 5.7% after 14 days ( $p < 0.05$ ) relative to the control group ( $n=8$ ) was observed for the daily Taxol group whereas Riminocelles did not incur significant weight loss neither were blood markers of toxicity elevated after acute administration ( $n=3$ ). The safety and efficacy of Riminocelles is statistically superior to Taxol. However, passive tumor targeting was not achieved and the tumor burden progressed quickly. Prior to further animal studies, the *in vivo* thermodynamic instability of the simple lipopolymeric micellular delivery system requires improvement so as to maintain and selectively deliver the fixed-ratio drug combination.

## Introduction

Paclitaxel (PTX) is a potent antineoplastic, microtubule-targeting drug used clinically against a wide variety of malignancies including ovarian, breast and non-small-cell lung cancer. The long standing commercial PTX formulation called Taxol® (Bristol-Myers Squibb) uses Cremophor EL® and anhydrous ethanol (1:1, v/v) as a solvent vehicle. The amount of Cremophor required to deliver an adequate dose frequently results in acute hypersensitivity reactions that therefore necessitates premedication with antihistamines and corticosteroids [1]. For this reason, numerous re-formulations of PTX based on nanoparticulate delivery systems have been developed to different extents [2], however to date only Abraxane® (albumin bound PTX) has reached ICH markets. The more pertinent concern is that chemotherapy with PTX often fails due to acquired or intrinsic drug resistance [3].

Multidrug resistance (MDR) has been described as the “thorniest obstacle” in developing improved systemic therapies for cancer [4]. Categorically, MDR is classically associated with the over-expression of ATP binding cassette (ABC) transmembrane proteins, particularly ABCB1/MDR1/P-glycoprotein (Pgp). These energy dependent efflux pumps extrude various structurally and mechanistically unrelated chemotherapeutics including Taxanes, Vinca alkaloids, Epipodophyllotoxins as well as modern rationally designed tyrosine kinase inhibitors [5] amongst other drugs to the extracellular environment thereby maintaining intracellular concentrations below effective cytotoxic levels [6]. Despite several compounds showing considerable activity *in vitro*, due largely to aberrant pharmacokinetic interactions leading to altered toxicity profiles there are currently no Pgp inhibitors (termed chemosensitizers) approved for clinical use [7]. Non-classical (non-transporter mediated) resistance involves evasion of cellular death through impairment of pathways controlling programmed cell death, as well as altered expression of enzymes responsible for detoxification and repair. Many of these alterations (e.g. inappropriate expression of oncogenes and loss of tumor suppressor gene function) are essential to neoplastic transformation itself. As these dynamics are complex and ill understood the contemporary strategy against MDR cancer involves the use of chemotherapeutics in combination. The rationale being that the MDR phenotype can be overcome (circumvented) through the action of multiple cytotoxic mechanisms acting in concert [4]. A strong correlation exists between the number of drugs used and successful cures [8]. Optimizing fixed-ratio drug combinations (FRDC) *in vitro* for synergistic interactions has emerged as a rational approach [9] to develop improved combination regimens, particularly when the drugs are co-formulated and specifically distributed to tumor tissue via systemically targeting nanoparticulate drug delivery systems (NDDS) [10-13].

The Rimonophenazine, clofazimine (B663) is a marketed anti-leprosy drug that has repeatedly demonstrated potent *in vitro* antineoplastic activity against a broad range of non-classical, intrinsically drug resistant tumor cell cultures including human hepatocellular, colorectal [14-16] and non-small-cell lung cancer [17]. In addition, B663 and derivatives have been shown *in vitro* to inhibit the action of Pgp [18-21]. The antiproliferative activity of Rimonophenazines appears multi-mechanistic, taking action at the plasma membrane [22], the mitochondria [23] and the nuclear level [24]. Rimonophenazines therefore possess huge therapeutic promise as broad-spectrum resistance circumventing agents. *In vivo* studies have demonstrated clofazimine given orally to be both safe and efficacious against

carcinogen induced sarcomas and mammary tumors [25] as well as human non-small cell lung carcinoma xenografts in nude mice [17]. Clinical investigations using clofazimine alone [26] or in combination with doxorubicin [27] against refractory metastatic hepatocellular carcinoma further support repositioning clofazimine as an anticancer agent in combination with Pgp substrates.

The therapeutic aim of this study was to increase the anticancer efficacy and decrease toxicity as compared to Taxol. To this end, Riminocelles™ was developed to co-formulate an *in vitro* optimized synergistic FRDC of PTX and B663. Riminocelles is a lipopolymeric (diacyl phospholipid-PEG) micelle that due to the size, zeta potential, low critical micelle concentration (CMC) value and *in vitro* stability (retention) under sink conditions is thought to offer prolonged systemic circulation, facilitating exploitation of the enhanced permeability and retention (EPR) effect [28-30]. This report describes the pre-clinical evaluation including acute toxicokinetics, repeat dose toxicity and anticancer efficacy of Riminocelles in parallel to Taxol® at an equivalent PTX dose. The potential utility of simple DSPE-PEG micelles for *in vivo* applications is discussed in light of the findings and with reference to recent thermodynamic understanding of the instability of such micelles in the presence of abundant albumin [31].

## Materials and methods

### Chemicals and reagents

An ELGA water purification unit (ELGA, Wycombe, UK) was used to produce 18 MΩ water from the municipal water supply. Cremophor EL, anhydrous ethanol, chloroform, methanol, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO). Paclitaxel (Fig. 1) was obtained from either Sigma Aldrich (St. Louis, MO, USA) for acute toxicity studies or from Hauser Pharmaceutical Services for efficacy studies. Clofazimine (Fig. 1) was a kind gift from Dr. J. F. O'Sullivan, Laboratories of the Medical Research Council of Ireland, Trinity College, Dublin, Republic of Ireland. LIPOID PE 18:0/18:0-PEG 2000 (1, 2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-[methoxy (polyethylene glycol)-2000] – DSPE-PEG 2000) and LIPOID S 75-3 Phosphatidylcholine were purchased from Lipoid GmbH (Ludwigshafen, Germany) after initially receiving samples as gifts.

### *In vitro* antiproliferative studies

Intrinsic Pgp expressing colorectal adenocarcinoma (COLO 320DM, ATCC CCL-220) cultured in RPMI media (with 10% foetal calf serum) were used at a seeding density of 5000 cells/well in 96-well plates. The antiproliferative effect of PTX and B663 alone as well as that of various fixed molar drug ratios of PTX and B663 (10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10) over a range of dose levels were determined in triplicate. The cells were incubated with the various drug/s for 7 days in 5% CO<sub>2</sub> at 37°C before performing the MTT assay to determine viability relative to vehicle (0.5% DMSO) treated controls. In

brief, 20  $\mu$ l of 5 mg/ml MTT in Phosphate Buffered Saline (PBS) was added to each well. After 4 hours incubation, the plates were subjected to successive centrifugation and PBS washing steps before drying the pellets. Finally 100  $\mu$ l of DMSO was added to each well and the plates were read spectrophotometrically using a universal microplate reader (ELx 800 UV) at 570 nm, using 630 nm as a reference. Dose response curves were plotted and IC<sub>50</sub> values determined using GraphPad (version 5.01). The antiproliferative data was appropriately transformed and captured into CalcuSyn software, version 2.0 (Biosoft, Cambridge). Using the software, the curves were fitted to a linear model using the median effect equation allowing for combination indexes (CI), as the measure of synergy, to be simulated for any fraction affect (*fa*) level using the combination index equation (where: additive activity=1; synergy<1; antagonism>1) of various FRDC at simulated *fa* values. In addition, the dose reduction afforded by the different FRDC was expressed as the percentage reduction in the IC<sub>50</sub> value compared to PTX used alone.

Prior to *in vivo* efficacy studies, a fixed molar ratio of 1:5 (PTX: B663) was screened against an additional human colorectal adenocarcinoma (HCT-15, ATCC CCL-225) cell line as a proof of potentiation. Neoplastic cells were seeded at a density of 2500 cells/well and were incubated in 5% CO<sub>2</sub> at 37°C with the FRDC for 3 days before performing MTT assays. The dose reduction afforded (being the benefit of synergy) was determined and used to compare to the COLO 320DM cell line.

#### Preparation of drug formulations

Taxol® was prepared fresh for each experiment by dissolving PTX in ethanol, sonicating briefly in a sonication water bath followed by adding an equal volume of Cremophor to make a 6 mg/ml PTX solution (protected from light and stored at -20°C). Prior to administration this solution was diluted with sterile saline (0.9 w/v NaCl) to produce a clear, colourless 1 mg/ml PTX solution.

Riminocelles™ was formulated in the Department of Pharmacology, University of Pretoria through the thin film hydration method. In brief, a pre-determined, optimised ratio of PTX and B663 were mixed together in büchi flasks with calculated volumes of the amphiphiles (DSPE-PEG 2000 and phosphatidylcholine) to produce the desired drug: drug ratio and total drug: amphiphile ratio. The organic solvents were evaporated off under reduced pressure <40 °C and desiccated overnight. The dried films were then hydrated with a pre-determined volume of 10 mM HEPES with the aid of a sonication water bath. The dispersions were filter sterilized through sterile, 25 mm, 0.2  $\mu$ m PTFE syringe filters. Aliquots were taken and the concentration of PTX and B663 within various replicates was quantitated using an optimised LC-MS/MS method. In brief, an Apollo C18, 5 $\mu$ m (150 x 4.6 mm) column with an isocratic mobile phase of 95% MeOH (0.1% formic acid, pH adjusted to 3.5 with ammonia hydroxide) was used with a flow rate of 1 ml/min for a total run time of 5 min. An AB Sciex 4000 Qtrap mass spectrometer was used to detect and quantify the analytes using multiple reaction monitoring in positive ion mode after first quantitatively optimizing the individual MS/MS transitions parameters for the best signal intensity. Replicate preparations were standardized through weighted pooling to a final synergistic FRDC of 1 mg/ml PTX (MW: 853.9) and 2.5 mg/ml B663 (MW: 473.4), therefore the prepared molar ratio of Riminocelles was 1: 4.5, PTX: B663. Riminocelles was stored at -20°C. For nude mice

studies, Riminocelles was shipped via DHL at -20°C to Charles River Laboratories (Ann Arbor, Michigan, USA) as 40 x 1 ml vials.

#### Acute *in vivo* toxicokinetic assessment

A total of sixty seven female BALB/c mice (weighing: 15.9 - 25.8 g) were acclimatized to the laboratory conditions of the University of Pretoria, Biomedical Research Centre for a week prior to experimentation. Mice were individually ear-tagged and fed standard rodent food and water *ad libitum*. All animal studies were prospectively reviewed and approval given by the Animal Use and Care Committee (AUCC) of the University of Pretoria (project # H10-09).

On Day 1 of the 14 day study, 12 animals were weighed facilitating the calculation of bolus injection volume for each specific mouse: Mouse weight (kg) x 10 mg/kg / 1 mg/ml = X (ml). Sterile Riminocelles [1 mg/ml PTX, 2.5 mg/ml B663] and Taxol [1 mg/ml PTX] were injected IV into the topically anaesthetised tail veins of 6 mice each at an equivalent PTX dose of 10 mg/kg using a 30-gauge needle over ~1 min using a 1 ml syringe. After administration the animals were observed daily for clinical evidence of toxicity and local tolerance. All surviving animals were weighed every second day for the duration of the study.

On Day 7, 3 mice were randomly taken from each of the two treatment 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. 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) and Gamma-glutamyl transpeptidase (GGT).

On Day 8 and 9, 55 mice were used in a pharmacokinetic and tissue distribution study to assess and compare the systemic exposure of the two PTX formulations. Five mice for both Riminocelles and Taxol were injected for each time period (30 min, 1, 3, 6 and 24 h). Five mice were treated with saline and euthanized after 24 h, serving as the source of drug naive matrix. Drug treated mice that were euthanized 24 h post administration were assessed for both toxicity markers and drug exposure. At euthanization, blood and organs (liver, spleen, kidney and fat tissue) were collected and stored at -70°C prior to accurate and precise quantitation using an optimized sample preparation and LC-MS/MS methods (unpublished). Plasma and tissue area under the curve ( $AUC_{0-24\text{ h}}$ ) as well as maximal concentrations ( $C_{max}$ ) and the terminal half-life ( $t_{1/2}$ ) were calculated and compared for each of the PTX formulations after intravenous bolus administration by non-compartmental analysis using PKSolver 2.0. The ratio between PTX and B663 in plasma and various tissues after administration of Riminocelles was compared to that originally encapsulated as a means to assess the *in vivo* functionality and stability of the NDDS.

On Day 14, the remaining 3 mice in each group that had been treated on Day 1 were euthanized, blood drawn and processed for toxicity markers. Through using this refined experimental

design: plasma toxicity biomarker profiles were collected for both formulations after 1, 7 and 14 days post administration (n=3); drug levels were determined at 5 time points from plasma and various tissues for both Taxol and Riminocelles groups (n=5), whilst reducing the animal numbers required.

#### Human tumor xenograft model

Forty female, athymic mice (Crl: NU-Foxn1<sup>nu</sup>) of age 8-9 weeks (weighing: 20.5 - 25.9 g) were acclimatized to the laboratory conditions of Charles River Laboratories (Ann Arbor, Michigan, USA). 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. The environment was controlled to a temperature range of 21±2°C and a humidity range of 30-70%. All drug treatments, daily clinical observations, tumor measurements and body weight determinations were carried out in these aseptic conditions. This experiment was conducted in compliance with all the regulations and guidelines of the National Institute of Health (NIH) and with the approval of Ann Arbor's Animal Care and Use Committee.

Human colon adenocarcinoma (HCT-15) cells that are known to intrinsically express high levels of Pgp [32] were purportedly obtained from Piedmont Research Center and were cultured using RPMI 1640 supplemented with 10% non-heat inactivated fetal bovine serum, 1% (1 M HEPES), 1% Penicillin-Streptomycin-Glutamine and 1% sodium pyruvate in 5% CO<sub>2</sub> at 37°C. The HCT-15 cells were detached and harvested from culture flasks using 0.25% trypsin/2.21 mM EDTA in HBSS. The HCT-15 cells with trypsin were washed with complete media and were collected through centrifugation at 300 g for 8 minutes at 4°C. After decanting the supernatant, cell pellets were combined and suspending in complete media. The viability of the HCT-15 cell suspension was determined with the trypan blue exclusion assay using a hemacytometer both before and after the inoculation period. Prior to implantation the cells were again centrifuged, collected and resuspended in 50% serum-free RPMI media and 50% Matrigel® to obtain a final cell concentration of 2.5x10<sup>7</sup> cells/ml. On Day 0, mice were implanted subcutaneously in the right axilla with 0.2 ml of the cell suspension (5x10<sup>6</sup> cells) using a 27-gauge needle and a 1ml syringe. During the inoculation period, the cell suspension was maintained on wet ice to minimize loss of viability and inverted frequently to maintain a uniform cell suspension. Treatment began once the tumors reach 100-200 mg (target 150 mg). Prior to initiation of treatment, animals were weight matched and assigned to respective groups such that the mean tumor burden in each group was within 10% of the overall mean. Mice were divided into five groups of 8 that included a daily (QDx7) and weekly (Q7Dx) dosing schedule group for both Riminocelles and Taxol as well as an untreated control. Mice were dosed according to their individual body weight on the day of treatment according to the formulae: Mouse weight (kg) x 10 mg/kg / 1 mg/ml = X (ml). As before, calculated volumes of Riminocelles [1 mg/ml PTX, 2.5 mg/ml B663] and Taxol [1 mg/ml PTX] were injected into tail veins such that a PTX dose of 10 mg/kg was administered.

#### Tumor measurements and efficacy endpoints

Tumor measurements were recorded three times weekly. Tumor burden (mg) was estimated from caliper measurements by the formula for the volume of a prolate ellipsoid: Tumor volume ( $\text{mm}^3$ ) = width<sup>2</sup> x length (mm) x 0.5, whilst assuming specific gravity to be 1 g/cm<sup>3</sup>, therefore  $\text{mm}^3 = \text{mg}$  [33]. Tumor growth delay (T - C value) was used as the primary efficacy endpoint, where T is the median time in days required for a particular treatment group to reach a predetermined size and C is the median time required for the control group to reach the same predetermined evaluation size [34]. Tumor growth delay was measured at an estimated tumour weight of 750 mg. The median times to this evaluation size for all study groups were first analyzed by application of the log rank (Kaplan-Meier) test to determine if any significant differences existed between groups. Upon identification that significant differences did exist, Bonferroni-Holm multiple comparisons post tests were performed to isolate the groups that differ from one another with a  $p < 0.05$  being considered as statistically significant.

Tumor-cell-kill and tumor-growth inhibition (%T/C value) was used as secondary efficacy endpoints. Tumor-cell-kill was calculated from the formula: The  $\log_{10}$  tumor-cell-kill total (gross) = [T - C value in days / 3.32 ( $T_d$ )], where  $T_d$  is the tumor-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 [35]. The number 3.32 is the amount of doublings required for a population to increase by an order of magnitude [34]. Tumor growth inhibition values (%T/C) were determined every second day through comparison of the median tumor burden for each group relative to the untreated control. Further, the time to euthanization at a tumor burden of >1g was considered for individual mice and used as an indicator of survival time.

#### Repeat dose toxicity assessment

As body weights of treated mice (in the efficacy study) were recorded three times weekly this served as a cost effective means to assess the repeat dose toxicity of the respective formulations in comparison to the untreated control group. Macroscopic necropsy was performed to identify possible target organs of toxicity and to note the occurrence of metastasis.

A one-way ANOVA was used to compare the weight differences between the various treatment groups and untreated control group. For specific time points, Dunnett's multiple comparison post tests were used to identify statistically significant treatment-related weight loss relative to the control group.

## Results

#### Determination of *in vitro* synergy

Dose response curves were plotted for each drug alone as well as for selected fixed molar ratio combinations (1:1, 1:2, 1:5, 1:10) of B663 and PTX (Fig. 2a). CalcuSyn software was used to perform median effect analysis and generate CI index values for actual and simulated  $f_a$  levels. 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. CI values for PTX:B663 ratios with a greater proportion of B663 were found to be synergistic ( $CI < 1$ ) at all simulated  $f_a$  levels (0.1- 0.9). The trend is for synergy to be greater at lower  $f_a$  levels (Fig. 2b). This observed synergy is responsible for the large percentage reduction in the  $IC_{50}$  value of PTX against COLO 320DM cultures. The  $IC_{50}$  value was reduced 83% from 42 nM (for PTX alone) to 7 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 for COLO 320DM cultures is 1353 nM. For HCT-15 cultures, the  $IC_{50}$  value was reduced 72% from 123 nM (for PTX alone) to 35 nM when used in a FRDC of 1:5 with B663, while the  $IC_{50}$  value of B663 for HCT-15 cultures is  $>2000$  nM.

#### Acute toxicokinetics

All IV injections were well tolerated during the first 5 minutes post administration and subsequent observation points. The animals in each group were monitored 3 times a day and weighed every second day for latent effects up to 14 days. Riminocelles was locally well tolerated at the injection site and no clinical signs of toxicity were evident throughout the entire observation period. There were no statistically significant differences between the weights on Day 0 and Day 14 for either formulation (data not shown). Haematology and clinical chemistry of terminal blood samples was unremarkable for the Riminocelles group. GGT was however present at all time periods (1, 7 and 14 days) for Taxol treated groups and although not significant an elevated level of AST is evident (Table 1).

The pharmacokinetic and tissue distribution study showed Riminocelles to be similar to Taxol in terms of PTX distribution to various tissues. The most substantial difference being a reduction in the plasma  $AUC_{0-24 h}$ ,  $C_{max}$  and half-life for Riminocelles whilst there is increased distribution of PTX to the liver, spleen and kidney compared to Taxol (Table 2). Comparative drug level analysis 30 minutes post administration demonstrates that the optimised loaded ratio of 1: 2.5 (PTX: B663, w/w) is not maintained in either plasma or any of the tissues suggesting rapid micelle disassembly. The plasma drug ratio after 30 minutes (Table 2) was 1: 0.4, whereas the drug ratio in fat tissue was 1:3.6 owing to different pharmacokinetic profiles and in particular rampant uptake of free B663 by fat tissue as is well established [36]. Macroscopic evaluation could not confirm specific 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 the B663 formulation.

#### Efficacy assessment

Treatment began on day 7 when the mean tumor burden was 160 mg (range, 151-168 mg). The control group reached an evaluation size (750 mg) after 16 days and were terminated due to tumor burden  $> 1$  g on Day 22. The activity of two different schedules of Riminocelles and Taxol against HCT-15 human xenografts is represented by the median tumor burden over time (Fig. 3), as this is the manner prescribed by the National Cancer Institute (NCI) for tumor-mass information [37]. It is immediately apparent that Riminocelles outperformed Taxol. The log rank statistic for all the survival curves was

significantly different ( $p < 0.001$ ). Treatment with Riminocelles following a schedule of QDx7 or Q7Dx2 produced statistically significant ( $p < 0.05$ ) growth delays (T-C) of 3.2 and 2.7 days respectively whilst treatment with Taxol did not result in any significant tumor growth delay (Table 3.). The tumor-volume doubling time ( $T_d$ ) was determined from the control group (whilst in exponential growth between 200-800 mg) to be 4.2 days and is within the historical range of the model. Tumor cell log kill (gross), assuming the initial growth rate is equal to the post treatment regrowth rate is reported in Table 3. A tumour-cell-kill of 0.23 was achieved for Riminocelles following a daily treatment schedule. A value  $> 0.7$  is considered active by the Drug Evaluation Branch of the Division of Cancer Treatment (NCI) and a value of greater than 2 is required to produce tumour regression in most models [37]. Tumor growth inhibition (%T/C values) at multiple time points from the day treatment started to Day 22 when the mice in the control group were terminated are shown in Fig. 4. The best tumor inhibition was 54% produced by Riminocelles following a daily schedule after 4 days of treatment. The NCI consider a %T/C  $\leq 42\%$  as significant antitumor activity [37]. In Fig. 5, the survival of individual mice is considered. In the QDx7 Riminocelles group 12.5% (1 of 8) of the population survived 31.3% longer than both the untreated control and the Q7Dx2 Taxol group that was shown not to increase the time to  $> 1$  g tumor burden compared to the untreated control.

#### Repeat dose toxicity

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 mild yellow coloration mimicking jaundice evident particular on the ears that can be attributed to clofazimine distribution to fat. Comparative weight changes of treated nude mice relative to the untreated control was used as an indicator of repeat dose toxicity. Statistical analysis of the percent body weight changes on Day 14 revealed a statistically significant difference ( $p < 0.05$ ) of 5.7% between the control group and the Taxol (QDx7) group using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post-test (Fig. 6). Regardless, as these weight changes were minimal, at this dosage both treatment regimens are considered well tolerated. Percent body weight loss at nadir and day of nadir are recorded in Table 3.

#### Discussion

Cancer accounts for upward of 8 million deaths each year [38]. Metastasis is largely responsible for cancer death - approximately 30% of patients have clinically detectable metastasis already at the time of diagnosis [39]. Surgical resection and traditional radiation being regional modalities have limited scope once the disease has metastasized. Therefore therapeutic interventions must be systemic (and targeting) in nature to tackle dissemination.

Taxol has been hailed as the best-selling anticancer drug in history [40]. Taxanes (including docetaxel) are commonly used in combination with various chemotherapeutics including anthracyclins,

platinum-based drugs, antimetabolites and biologicals (monoclonal antibodies). The optimal combination partner/s and dosing regimens for various cancers remains unclear and current polychemotherapeutic regimens (empirically developed in late stage clinical trials) do not fully exploit the potential synergistic actions (and therefore dose reduction) offered through rationally combining drugs in optimised fixed-ratios [9]. Apart from dose-limiting systemic toxicities, the major reason for the relatively poor prognosis of cancer is resistance to existing chemotherapeutic treatments and therefore developing agents that circumvent MDR mechanisms is of paramount importance.

In this R&D study, the strategic pre-clinical development plan employed a synergistic fixed-ratio drug combination encapsulated within a nanoparticulate drug delivery system (the FRDC-NDDS approach) to improve upon the therapeutic index of Taxol. Although numerous novel PTX reformulations have and continue to be developed, few have the specific agenda of overcoming MDR through co-formulation of a drug combination including a Pgp inhibitor. Contemporary innovations to this end include the use of Pgp inhibitors as either an active excipient in the assembly of the carrier [41] or as a co-encapsulated drug [42-44] and [45, 46] who similarly made use of DSPE-PEG 2000 based micelles. Riminocelles is unique in comparison in that PTX and B663 have been co-encapsulated at an *in vitro* optimized synergistic ratio. B663 inhibits Pgp and further possesses an antiproliferative multi-mechanism ensuring activity against a broad range of cancers and is therefore an ideal MDR circumventing combination partner. A lipopolymeric (DSPE-PEG 2000) micelle that is capable of entrapping multiple hydrophobic drugs within its core and is purported to offer stable prolonged circulation (facilitating passive targeting) was chosen as the delivery system.

Pre-clinical studies were designed and performed so as to directly compare the therapeutic potential of Riminocelles to Taxol at an equivalent PTX dose of 10 mg/kg: The IV suitability, local tolerability and lack of elevated toxicity markers produced by Riminocelles in contrast to Taxol has been demonstrated in an acute toxicity study with a 14 day observation period; the plasma and tissue distribution of Riminocelles and Taxol over 24 h post administration demonstrated that the distribution of PTX was similar for the two formulations. Of paramount importance is that the integrity of the fixed-ratio combination of Riminocelles was quickly lost, strongly suggesting rapid micelle disassemble *in vivo*; concerning repeat dose toxicity, daily treatment with Taxol for a week resulted in significant weight loss after 14 days relative the untreated control. To the contrary, treatment with Riminocelles did not incur significant weight loss; of note, the efficacy of Riminocelles was shown to be statistically superior to that of Taxol using a Pgp expressing drug resistant human xenografted nude mouse model.

Taken together, these results point to the translational pitfall of this popular drug delivery platform and indicate that the minor gain in efficacy attained can be attributed to the addition of B663 and not to any pharmacokinetic advantage offered through formulation within lipopolymeric micelles. Passive tumour targeting of the FRDC could not be realized as the DSPE-PEG 2000 micelles quickly disassembled *in vivo* owing to a lack of strong intermolecular forces between the amphiphiles and adsorption by highly abundant albumin [31]. Therefore, the lipopolymeric micelles act merely as a convenient solubilizing system for hydrophobic compounds and not as a passive accumulating drug delivery system. The original studies upon which the *in vivo* stability/longevity of DSPE-PEG micelles is based did not assess the *in vivo* integrity of the micelles but rather monitored the biodistribution of a

radio-labelled lipid (phosphatidylethanolamine residue)-derivatized protein cargo [47] and essentially radio-labelled unimers [48] that would readily adsorb onto albumin. Vakil and Kwon, 2007 [49] were seemingly first to report on the destabilizing effect of albumin on “PEG-phospholipid micelles” and demonstrated the core structuring benefit of cholesterol. Kastantin et al. [31] reported a thermodynamic understanding of the *in vivo* instability of DSPE-PEG 2000 micelles in the presence of albumin and showed DSPE-PEG 2000 to exist in equilibrium as micelles, as unimers and bound to albumin. The latter being strongly favoured at physiological temperatures. This finding in conjugation with the current illustration of the *in vivo* instability of DSPE-PEG 2000 micelles bears repeating for those with a true translational interest.

In conclusion, a novel cremophor free PTX drug delivery system (requiring no premedication nor special –non PVC infusion systems) that outperforms Taxol in terms of safety and efficacy has been developed and pre-clinically evaluated. Advantageously, all the components (excipients and drugs) used in the assembly of the Riminocelles are already individually approved for medicinal use, in theory promoting accelerated translation to the clinic. The potential clinical role of clofazimine as a resistance circumventing addition to PTX regimes is clear. Employing a synergistic FRDC undoubtedly improves the therapeutic index of PTX. The strategy of employing the FRDC-NDDS approach is clearly beneficial. However, the simple lipopolymeric micellular NDDS did not function as intended owing to instability *in vivo*. DSPE-PEG 2000 micelles do not offer prolonged circulation of encapsulated drugs and therefore does not offer targeting via the EPR effect. Stability improvements (perhaps protein or polymeric scaffolding) aimed at increasing the activation barrier for desorption are called for. Further pre-clinical work should focus on intelligent, tumour targeting NDDS that encapsulate diverse synergistic FRDC so as to improve patient outcomes.

**Conflict of interest**     The authors declare no conflict of interest

**Acknowledgements**     Financial support for this study was provided by BioPAD (BPH 004) and the Department of Pharmacology, University of Pretoria.

**Declaration of ethical standards**     The experiments in this work comply with the current laws and standards of the countries in which they were performed.

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## TABLES AND FIGURES

**Table 1** Plasma toxicity biomarkers 1, 7 and 14 days after acute IV injection of Riminocelles and Taxol at an equivalent PTX dose of 10 mg/kg (n = 3)

	Riminocelles			Taxol		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
<b>Clinical chemistry</b>						
ALT (IU/l)	31.2 ± 5.1	26.3 ± 2.50	22 *	28.6 ± 2.7	33.3 ± 18.2	26.7 ± 6.4
AST (IU/l)	89.2 ± 10.8	92.7 ± 28.7	71 *	113.4 ± 32.0	127.7 ± 16.4	97.3 ± 12.1
GGT (IU/l)	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	<18	<18	<18	<18	<18
<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 ± 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 low volume of blood drawn, replicate measures could not be attained

**Table 2** Difference in the tissue exposure of PTX between Riminocelles and Taxol (expressed as the n-fold change of Taxol) as well as the functioning of Riminocelles in terms of fixed-ratio maintenance

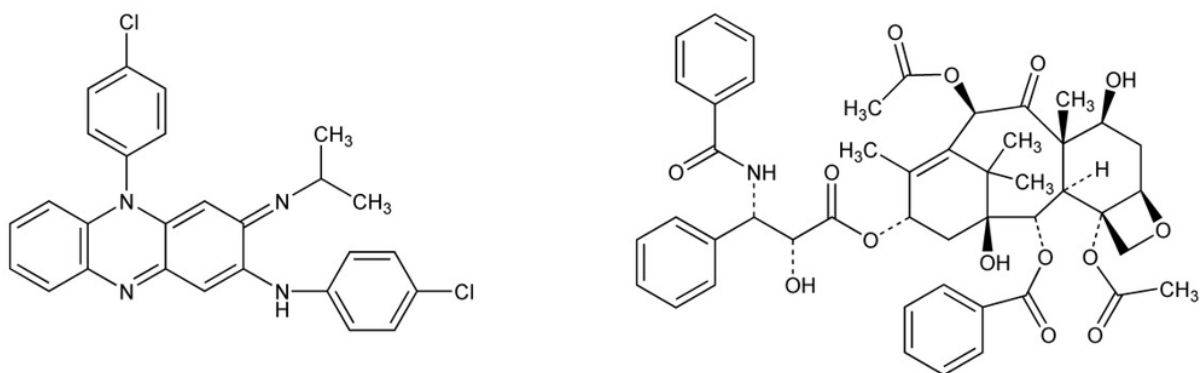
<b>Tissue</b>	<b>AUC<sub>0-24 h</sub></b>	<b>C<sub>max</sub></b>	<b>t<sub>1/2</sub></b>	<b>PTX: B663 at 30 min</b>
<b>Plasma</b>	0.56	0.48	0.73	1: 0.4
<b>Liver</b>	1.28	1.32	1.20	1: 0.7
<b>Spleen</b>	1.22	1.05	1.16	1: 0.9
<b>Kidney</b>	1.13	0.99	0.84	1: 0.7
<b>Fat</b>	0.89	0.82	1.00	1: 3.6

**Table 3** Summary: Safety and efficacy of Riminocelles in comparison with Taxol ( $n=8$ )

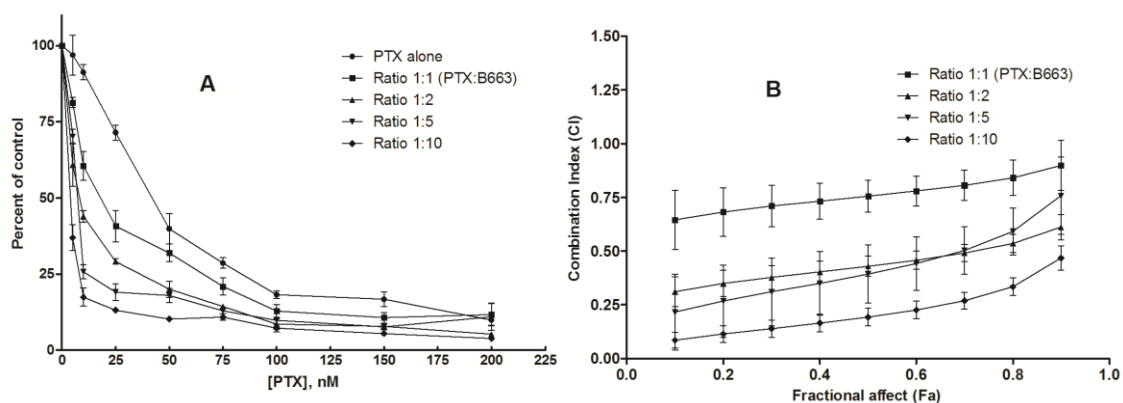
Group	Dose <sup>a</sup> (PTX) <sup>b</sup> (B663)	Schedule	Route	% Body wt. loss at nadir (day of nadir)	Rx related deaths	Tumor growth delay (days)	Tumor- cell-kill
Control	NA	NA	NA	0.4 (11)	0/8	NA	NA
Taxol	10 mg/kg <sup>a</sup>	QDx7	IV	3.5* (14)	0/8	0.5	0.04
Taxol	10 mg/kg <sup>a</sup>	Q7Dx2	IV	+	0/8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

+ Weight increases

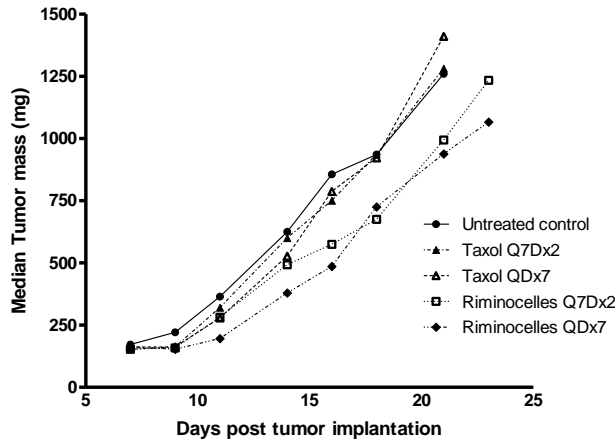
\* $p<0.05$



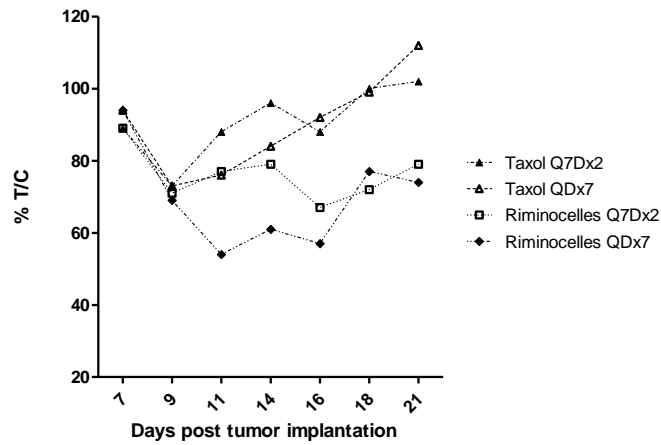
**Fig. 1** Chemical structure of clofazimine, B663 (left) and paclitaxel, PTX (right)



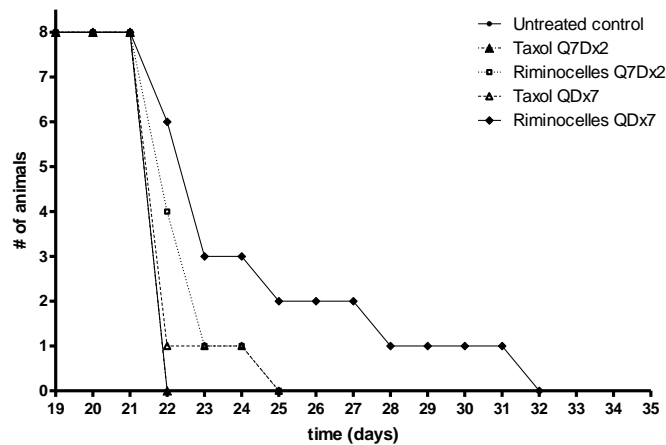
**Fig. 2** Synergistic *in vitro* antiproliferative activity of PTX alone and selected FRDC of PTX and B663 showing a) Dose response curves after 7 day drug incubation period (MTT assay); The antiproliferative effect of B663 alone is not displayed ( $IC_{50} = 1353$  nM) b) Fa-CI plot: Simulated CI index values as a function of the fraction affected (Fa) where  $CI < 1$  indicates synergy. The effect of PTX: B663 (10:1, 5:1, 2:1) were antagonistic ( $CI > 1$ ).



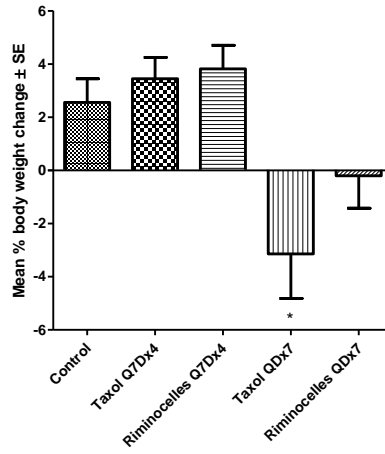
**Fig. 3** Median tumor burden-time profile of HCT-15 subcutaneous xenografted human tumors in nude mice. Mice were treated with 10 mg/kg PTX as either Riminocelles or Taxol following two different schedules ( $n=8$ )



**Fig. 4** Tumor growth inhibition normalized to the untreated control over time expressed as % T/C



**Fig. 5** Time to tumor burden of 1000 mg for individual mice. The survival of Taxol following a Q7Dx2 dosing schedule was no different than the untreated control



**Fig.6** Percent body weight change ( $n=8$ ) on Day 14 after dosing tumor bearing mice with Riminocelles and Taxol (10 mg/kg PTX). One-way ANOVA with Dunnett's multiple comparison post-test. \* $p<0.05$