

# Reversal of Chemoresistance in Ovarian Cancer Cells by the Liposomal Co-Delivery of MDR Inhibitors and Paclitaxel

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## ABSTRACT

Ovarian cancer is one of the most lethal gynaecologic malignancies. This can be attributed to the emergence of multidrug resistance in the clinic due to the over-expression of drug-efflux pumps such as P-gp. To overcome this, we have developed a liposomal platform for the co-delivery of potent P-gp inhibitors with chemotherapeutic drugs.

PEGylated liposomes composed of eggphosphatidylcholine, cholesterol and DOTAP loaded with equimolar amounts of tariquidar (XR) and paclitaxel (PCT) were prepared by the thin film hydration method followed by extrusion. The cellular effects of the formulations were evaluated using the holographic time-lapse cytometer HoloMonitor<sup>®</sup> M4 and iCyte<sup>®</sup> laser scanning cytometer

In vitro cytotoxicity experiments showed that the drug co-loaded formulation was able to effectively overcome resistance to PCT even at low PCT doses. Using live-cell imaging, paclitaxel-induced arrest of cell cycle progression and onset of apoptosis following this mitotic catastrophe was visible from 1.5  $\mu$ M to 50 nM only with the co-loaded liposomes. The cell cycle arrest was visualized using time-lapse phase holographic imaging which showed the increasing incidence of mitotic cell cycle arrest over time providing further evidence of this formulation-induced microtubule dysfunction.

## INTRODUCTION

Ovarian cancer is known to be one of the most lethal gynaecologic malignancies. The current standard of care comprises of debulking surgery followed by adjuvant chemotherapy with taxane and platinum-based drugs. Mortality rates for ovarian cancer have not drastically been improved in almost 40 years. In a vast majority of cases, this can be attributed to the development of multi-drug resistance (MDR) due to the overexpression of P-gp drug efflux pumps.<sup>1</sup> Very high doses of drugs are subsequently required to induce therapeutic effects. There is thus an urgent need to address these issues.

Tariquidar (XR), a 3<sup>rd</sup> generation P-gp inhibitor shows great potential because of its potent anti-MDR effects and low toxicity.<sup>2</sup> However, it needs to be specifically delivered to tumor cells thereby circumventing P-gp expressing normal organs. The co-administration of XR with a chemotherapeutic drug such as paclitaxel (PCT) could thus allow for the reduced extrusion of PCT. PCT belongs to the taxane drug family and is an antineoplastic agent that specifically targets the microtubule assembly of the cell.<sup>3</sup> It induces stabilization of the microtubules which subsequently initiates apoptosis through a number of pathways.<sup>4</sup> Long et al. were one of the first to show that paclitaxel specifically inhibits mitotic spindle assembly thereby preventing cell cycle progression to G1.<sup>5</sup>

We therefore hypothesized that the liposomal co-delivery of XR and PCT would represent an effective approach for the reversal of chemoresistance by circumventing many of the drug delivery issues associated with these drugs, thereby allowing for cancer cell-specific cytotoxicity.

## EXPERIMENTAL METHODS

### *Preparation and characterization of liposomes*

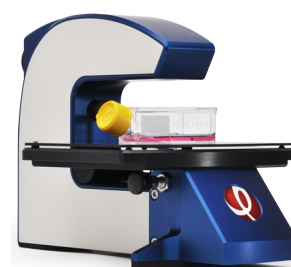
Liposomes composed of eggphosphatidylcholine, cholesterol, DOTAP and DSPE-PEG2000 were prepared by the thin film hydration method followed by extrusion through 200 nm polycarbonate membranes. The drugs, tariquidar (XR) and paclitaxel (PCT) were included (in equimolar amounts) in the lipid film at a 1% (w/w) ratio to the total amount of lipid. Following extrusion, the non-encapsulated drug was removed by syringe filtration of the formulation through 0.22  $\mu$  membranes. Liposomal drug concentrations were determined using reverse phase high performance liquid chromatography (HPLC). A mixture of 10 mM ammonium acetate buffer (pH 4) and acetonitrile was used as the mobile phase. The size of the liposomes was characterized using transmission electron microscopy (TEM) with a uranyl acetate stain as well as dynamic light scattering while the zeta potential was measured using a zeta potential analyzer.

### *In vitro experiments*

All experiments were carried out using a taxol-resistant SKOV3 ovarian cancer cell line (SKOV3-TR). For in vitro cytotoxicity experiments, 3000 cells were seeded per well in 96-well plates 24 hrs prior to experiments. The various liposomal formulations were then incubated with cells for 24 hrs and washed off. Cell viability was measured after a further 24 hrs

using the Promega cell viability assay. Similarly, for the analysis of apoptotic activity using the iCyte, following treatment with the formulations, the cells were stained with Hoechst, Yo-Pro and propidium iodide. For the analysis of cell cycle distribution, the cells were gated into the G1, S and G2 phases based on their DNA content.

For analysis using the HoloMonitor M4 cells were seeded into a T-25 flask and imaged at 5 minute intervals for 24 hrs. The drug co-loaded liposomes (LP (XR,PCT)) were then added at the required concentrations, and imaging was continued at 5 minute intervals for 48 hrs.



*HoloMonitor M4 – a holographic time-lapse cytometer*

## RESULTS AND DISCUSSIONS

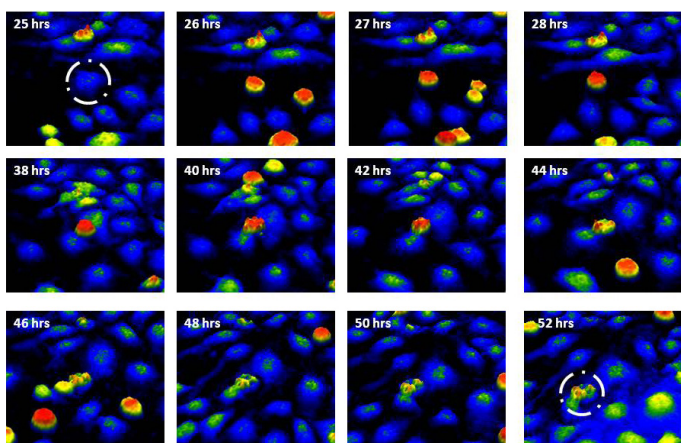
All the liposomal formulations exhibited a homogeneous size distribution of approximately 200 nm with a zeta potential of approximately 30 mV. HPLC analysis showed that the concentration of encapsulated drugs was about 23-24  $\mu\text{M}$  for both drugs for a 5 mg/mL liposomal lipid concentration.

The cytotoxic effects of the liposomal formulations were evaluated on SKOV3-TR cells. Even though free PCT and liposomal PCT could not induce cell death even at high concentrations of PCT (1.57 $\mu\text{M}$ ), the co-loaded formulation LP (XR,PCT) was able to effectively reverse PCT resistance causing significant cell death even at PCT doses as low as 50 nM. As expected, a similar effect was seen on the analysis of apoptotic activity showing enhanced formulation-induced apoptosis from 1.5  $\mu\text{M}$  to as low as 50 nM LP (XR,PCT). On analysis of cell cycle distribution, LP (XR,PCT) was shown to specifically inhibit progression to the G1 phase providing evidence of PCT-mediated microtubule dysfunction.

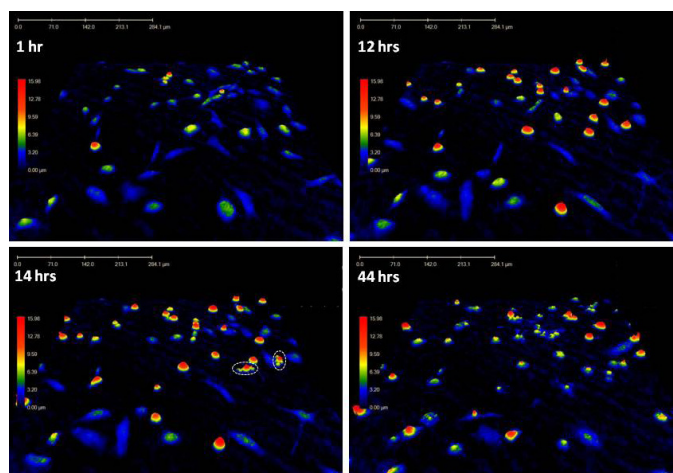
We then decided to further investigate the cytotoxic effects of the LP (XR,PCT) formulation on SKOV3-TR cells using the HoloMonitor M4. Until now, all our aforementioned methods constituted the analysis of the end-point after a 48-hour time period. It was therefore imperative to carry out a time course analysis in order to better understand the kinetics of the formulation-induced anti-cancer effects.

We carried out a preliminary study by treating the cells with a low concentration of 50 nM LP(XR,PCT) in order to be able to capture the paclitaxel-induced cell cycle arrest preceding apoptosis. As is shown in Figure 1, we were able to clearly visualize the arrest of cell cycle progression characterized by the presence of 'rounded' mitotic cells (shown in red) which were unable to complete mitosis (24 hrs after addition of formulation). Due to treatment with a low concentration of formulation, this mitotic arrest was seen up to 44 hrs after which the cell proceeded to the apoptotic phase (as seen in the 52 hr time point image with the cell circled in white in Figure 1).

Following this, we repeated the HoloMonitor study using a higher LP (XR,PCT) concentration of 200 nM. As expected due to the higher drug concentration, the cell population under analysis showed an increase in the rate of mitotic cell cycle ar-



**Figure 1.** HoloMonitor M4 images showing mitotic arrest of SKOV3-TR cells induced by 50 nM LP (XR,PCT).



**Figure 2.** HoloMonitor M4 analysis of SKOV3-TR cells on treatment with 200 nM LP (XR,PCT).

rest. As seen in Figure 2, this cell cycle arrest was evidenced as early as 12 hrs on treatment with the formulation. In 14 hrs, a few of the cells even proceeded to apoptosis (circled in white in Figure 2). By the 44 hr time point, the majority of the cell population had undergone apoptosis further highlighting the efficacy of the LP (XR,PCT) formulation.

## CONCLUSIONS

Uniform liposomes co-loaded with XR and PCT were prepared and characterized. The LP (XR,PCT) co-loaded formulation was able to effectively overcome resistance to PCT in SKOV3-TR cells even at low PCT doses resulting in enhanced cytotoxic effects. The co-loaded liposome was shown to arrest cell cycle progression specifically to the G1 phase providing evidence of paclitaxel-mediated microtubule dysfunction. Using time-course holographic imaging studies, this cell cycle arrest followed by progression to apoptosis was clearly visualized and confirmed.

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