

This article describes an investigation on the use of a combination therapy administered within polymeric nanoparticles in order to overcome Multiple Drug Resistance (MDR), one of the most challenging threats to survival in the battle against cancer.

A Multi-Functional Polymeric Nanoparticle Strategy for Modulation of Drug Resistance in Cancer

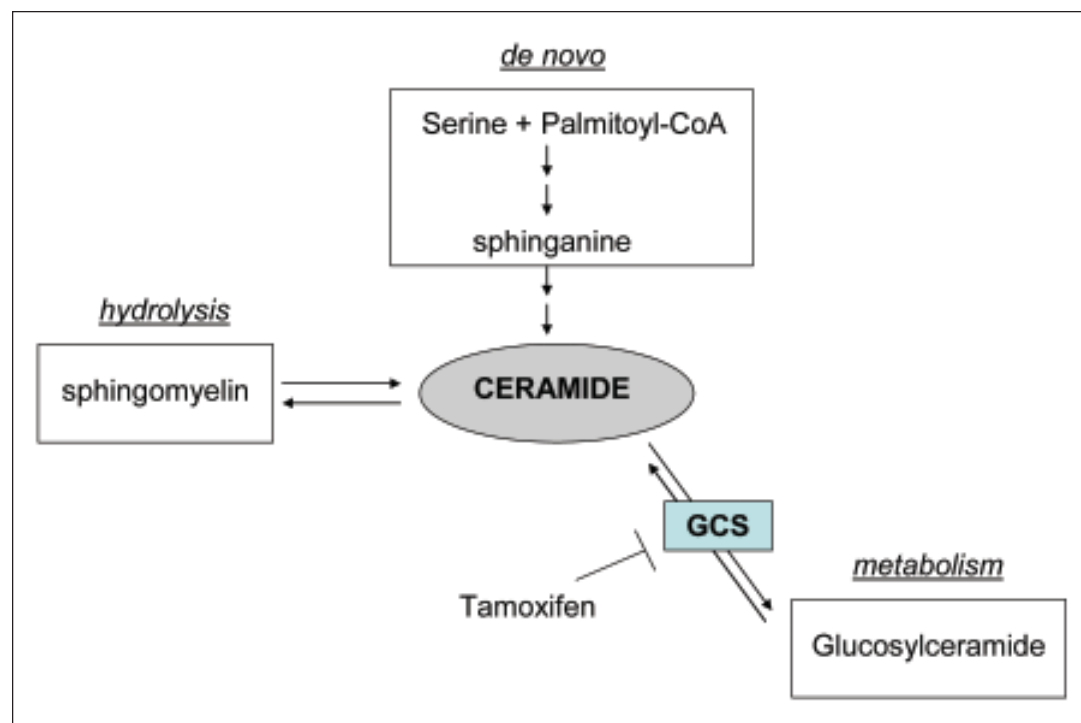
by Lilian E. van Vlerken and Mansoor M. Amiji

Introduction

In the battle against cancer, the development of Multiple Drug Resistance (MDR) poses one of the most challenging threats to survival, and is commonly found to be the reason for tumor persistence despite invasive chemotherapy. MDR refers to a cross-resistance to structurally and functionally unrelated drugs, thereby rendering the tumor unresponsive to most chemotherapeutic options. Chemo-resistance can generally result from either of two means, by a physical impairment to drug delivery to the tumor,¹ such as poor absorption, increased metabolism/excretion, or poor diffusion of systemically-administered drugs into the tumor mass, or more chal-

lengingly, through intracellular mechanisms in the cancer cell itself.² Alterations in the intracellular machinery of cancer cells is commonly implicated in the development of MDR, and often more than one mechanism, either simultaneous or sequential, may be responsible for development of the resistant cell phenotype.³ Initially, the ATP-dependent drug efflux transporters, which included P-glycoprotein, were identified as the sole basis for MDR,⁴ leading to tremendous therapeutic development efforts aimed at blocking the efflux transporters. Unfortunately, the preclinical and clinical results from this strategy have not been encouraging. This strengthened the idea that MDR in cancer is in fact due to other mecha-

Figure 1. Schematic illustration of ceramide synthesis and metabolism.



nisms besides drug efflux, and so in recent years, studies are being performed to establish and potentially exploit other mechanisms by which the MDR phenotype develops. Of this, the role of DNA repair following damage through topoisomerase I and II activity and neutralization of electrophilic drugs by glutathione-S-transferase have been reported as mechanisms whereby the cancer cells also develop chemoresistance.³ In addition, modulation of programmed cell death (*apoptosis*) following chemotherapeutic stress has emerged with clear importance as a strategy whereby cancers become chemoresistant. Deregulation of several key apoptosis modulating factors has been described in various experimental cases of MDR,⁵ including functional up-regulation or overexpression of anti-apoptotic mediators such as p21, Bcl-2, and Bcl-XL, and/or down-regulation of the classic oncogenic mediator, p53. As a result, MDR modulation strategies are increasingly looking away from the ABC-transporter paradigm and toward modulation of cellular apoptotic signaling. Several apoptosis modulating strategies (e.g., protein tyrosine kinases PKI166 and ST1571, Bcl-2 antisense such as G-3139, and retinoids 9-cis-RA and AM-580) are currently in clinical trials, and their efficacy in MDR modulation is largely under preclinical and clinical investigation.

Ceramide (CER), a naturally occurring sphingolipid, is derived intracellularly by hydrolysis of the lipid sphingomyelin, or by *de-novo* synthesis through N-acylation of sphinganine⁶ - *Figure 1*. Accumulation of endogenous CER, produced either by hydrolysis or *de novo* formation, is known to result in response to several stimuli, including stress, regulating apoptosis and cell cycle arrest, where CER functions as a second messenger in the signaling cascade that initiates these responses.^{6,7} In fact, studies have shown that administration of exogenous CER analogs, particularly C2- and C6-ceramide, encourages cell death by apoptosis and inhibition of tumor growth in several tumor models.⁸ In the cell, CER can subsequently be further metabolized by the enzyme Glucosylceramide Synthase (GCS) to yield glucosylceramide (gluCER), a glycosylated form of CER that does not have pro-apoptotic activity.⁹ Several MDR tumor cell lines have exhibited elevated levels of non-cytotoxic gluCER and corresponding elevated levels of GCS, and clinical studies have noted elevation of gluCER levels in tumor specimens of breast cancer and melanomas that were poorly responsive to chemotherapy.⁹ These findings not only suggest the importance of CER in the mediation of the cytotoxic response to anti-tumor chemotherapeutics, but also they suggest that inhibition of apoptotic signaling may be an important mechanism whereby tumors develop MDR.

While the development of MDR poses a great threat to survival of cancer patients, drug delivery to solid tumors in and of itself is a significant challenge that also determines survival outcome. A major barrier to successful anti-cancer therapy is the challenge of delivering the required therapeutic concentration to the tumor site while minimizing undesirable side effects resulting from systemic administration. Site-specific drug delivery systems increase the therapeutic benefit by delivering a greater fraction of the dose at the

target site, which minimizes the amount of therapeutic that accumulates at non-specific targets. Drug delivery throughout the tumor mass is crucial for the treatment to be effective since residual cancer cell survival can promote re-growth and often becomes the cause for drug resistance.¹⁰ Physical hurdles posed by solid tumors greatly hinder chemotherapeutic drugs from entering and/or traversing throughout the tumor mass, thereby resulting in an ineffective treatment. Nanoscale drug carriers, such as liposomes, micelles, dendrimers, and polymeric nanoparticles, can bypass these hurdles by taking advantage of unique physiologic parameters of the tumor mass, termed the enhanced permeability and retention (EPR) effect,¹¹ to greatly improve drug delivery to and throughout the tumor mass.

Biodegradable polymers such as poly(epsilon-caprolactone) (PCL) are useful materials to formulate drug delivery carriers for tumor targeted delivery. Biocompatibility and degradation methods of these polymers have been widely studied,¹² and found to be non-toxic, leading to the US FDA approval and acceptance for medical applications. Additionally, these polymers offer an advantage for drug delivery, whereby they efficiently encapsulate hydrophobic compounds, and slow degradation of the particle allows for extended release of the drug.¹³ Surface modification of the nanoparticles with a poly(ethylene oxide)-poly(propylene oxide) triblock copolymer (PEO-PPO-PEO, Pluronic[®]) improves the stability of the nanoparticle in the aqueous environment of the body, while decreasing immune activation, repelling plasma proteins and decreasing reticulo-endothelial uptake leading to an increase in circulation time and passive tumor targeting by the enhanced permeability and retention effect. Previous studies from our group have shown that paclitaxel (PTX)-containing PEO-PCL nanoparticles remain stable *in-vivo*, and retain their Pluronic[®] surface layer to increase the circulating half-life of PTX from a fraction of an hour to 25.3 hours, alongside an 8.7-fold higher tumor drug concentration.¹⁴

The purpose of this work was to overcome MDR in a model of human ovarian cancer through a combination therapy administered within long-circulating polymeric nanoparticles. The combination therapy consists of either C₆-ceramide (CER) or the GCS inhibitor tamoxifen (TAM), aimed to restore the defaults in apoptotic signaling, along with a pro-apoptotic chemotherapeutic drug paclitaxel. The aspect of this therapy is to overcome MDR through a multi-pronged approach that includes: (1) restoration in the defects in apoptotic signaling and (2) enhancement of drug delivery to the tumor site and by delivering the drugs intracellularly, thereby potentially avoiding P-glycoprotein-mediated drug efflux. Few groups have investigated the use of nanoparticles in the treatment of MDR, and those that have focused on facilitating the delivery of chemotherapeutic drugs past the P-glycoprotein pump, thereby evading drug efflux and leading to enhanced chemosensitivity. However, to date, the use of nanoparticles has not been investigated as a therapeutic approach to overcome alternate, or simultaneously multiple mechanisms of MDR, supporting the novelty of the described therapeutic approach.

Materials and Methods

Nanoparticle Fabrication and Characterization

Poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles were prepared by controlled solvent displacement in an acetone-water system with a 20% (w/w) surface modification with a poly(ethylene oxide)-poly(propylene oxide) triblock copolymer, Pluronic® F-108 NF grade. Nanoparticles were loaded individually at 10% (w/w) PTX, 20% (w/w) C₆-CER, or 20% (w/w) TAM. For intracellular trafficking studies, PTX-loaded nanoparticles were supplemented with 0.1% w/w rhodamine-paclitaxel. Nanoparticles were analyzed for size on a Brookhaven ZetaPlus particle analyzer and visualized by Scanning Electron Microscopy (SEM) on a at 5,000x magnification under an accelerating voltage of 3kV.

Cell Culture and Treatment

Human ovarian carcinoma cells, SKOV3, and their MDR phenotype, SKOV3_{TR}, were kindly provided by Dr. Michael Seiden (Massachusetts General Hospital, Boston, MA). The SKOV3_{TR} culture was developed by prolonged exposure of increasing concentrations of paclitaxel, and maintained in 0.2 µM paclitaxel to uphold the MDR phenotype. Cells were subjected to dose-response treatments of the individual drugs and drug combinations in serum-supplemented medium either as free drugs (solution) or encapsulated within PEO-PCL nanoparticles. Culture medium was used as a negative control (0% cell death) and 50 µg/mL poly(ethyleneimine) in medium was used as a positive control (100% cell death). Treatment proceeded for six days undisturbed at 37°C in a humidified chamber at 5% CO₂, after which remaining cell viability was measured by the MTS assay.

Intracellular Drug Trafficking and Quantitation of Intracellular Drug Levels

To quantitatively determine the amount of intracellular PTX accumulation resulting with or without the nanoparticle delivery system, PTX loaded PEO-PCL nanoparticles were manufactured as previously described with the addition of ³H-PTX at 1.5 µCi/mg unlabeled drug. SKOV3 and SKOV3_{TR} cells were allowed to adhere in six well plates at 1 x 10⁵ cells/well, and treated with a 0.1 µM dose of PTX for six hours at 37°C in a humidified cell culture incubator. Following the treatment period, cells were washed three times, lysed with 1 mL of lysis buffer, and collected into scintillation vials. Each sample received 10 mL Scintisafe® scintillation fluid per 1 mL lysis buffer, and was left to quench for two hours in the dark.

Following this, counts per minute of the ³H were collected on a α/β scintillation counter. To determine the total amount of protein in 1 x 10⁵ cells for each cell type, cells were lysed in parallel for extraction and quantitation of total protein. The results are expressed as % of dose accumulated intracellularly per mg of total protein.

Measurement of Apoptotic Activity

To measure the degree of apoptosis in SKOV3_{TR} cells following treatment with PTX alone and PTX + CER, apoptosis was

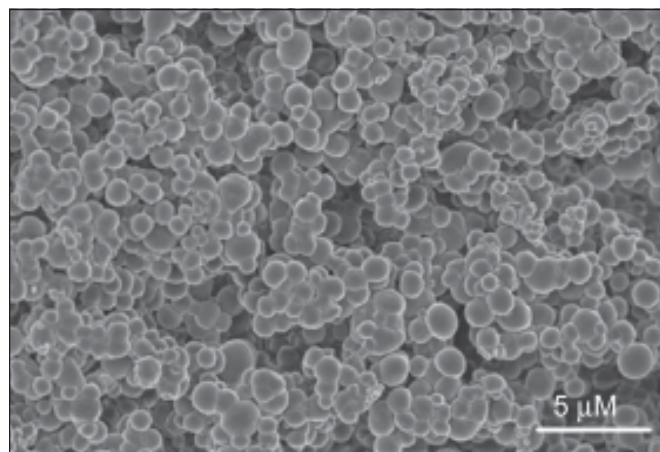


Figure 2. Scanning electron micrograph of poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles. (Scale bar represents 5 µM).

measured using a commercial apoptosis assay kit that stained apoptotic cells using Yo-Pro-1® and propidium iodide (PI). SKOV3 and SKOV3_{TR} cells were allowed to adhere into 96-well optical quality plates at a density of 2x10⁴ cells/well, and subjected to treatments with PTX, CER, or PTX + CER at varying doses for 12 hours. Following the treatment period, cells were stained for apoptotic activity and measured by *in-situ* cytometric analysis of live cells by simultaneous Laser Scanning Cytometry (LSC) and epifluorescent microscopy. Yo-Pro and PI were excited at 488 nm by an argon laser and absorbed at 515 to 545 nm and 600 to 635 nm respectively. Each sample scan was repeated four times, all treatments were run in triplicate, and the entire set up and analysis was repeated once more at a later date.

Results and Discussion

Using the solvent displacement method, optimized in our lab, PEO-PCL nanoparticles were formed in a reproducible manner with a uniform spherical appearance and a mean diameter of around 210 nm - *Figure 2*. The encapsulation efficiency of PTX, CER, and TAM was found to be more than 95% at the added concentrations in PEO-PCL nanoparticles. Dose-response studies on the SKOV3 and SKOV3_{TR} lines against PTX verified the highly drug-resistant nature of the MDR line, where PTX IC₅₀ was more than 100-fold higher at 1.08 µM (versus 0.008 µM for the SKOV3 cells), as demonstrated by the far right-shifted dose response curve - *Figure 3*. In addition, the MDR phenotype of this cell line was further characterized by the presence of both P-glycoprotein and GCS, which were not expressed by the SKOV3 cells (data not shown). Modulation of the MDR nature will result in chemosensitization against PTX, causing the far-right shifted dose-response curve to shift back toward that of the drug-sensitive SKOV3 cells. *Figure 3a* shows that the co-therapy of PTX with CER (at a consistent dose of 10 µM) on the SKOV3_{TR} cells in fact shifts the dose-response curve slightly to the left. Chemosensitization with this combination treatment is seen, for example, whereby a 1 µM dose of PTX kills merely one-third of the MDR population (65.6 ± 2.2% survival), but the

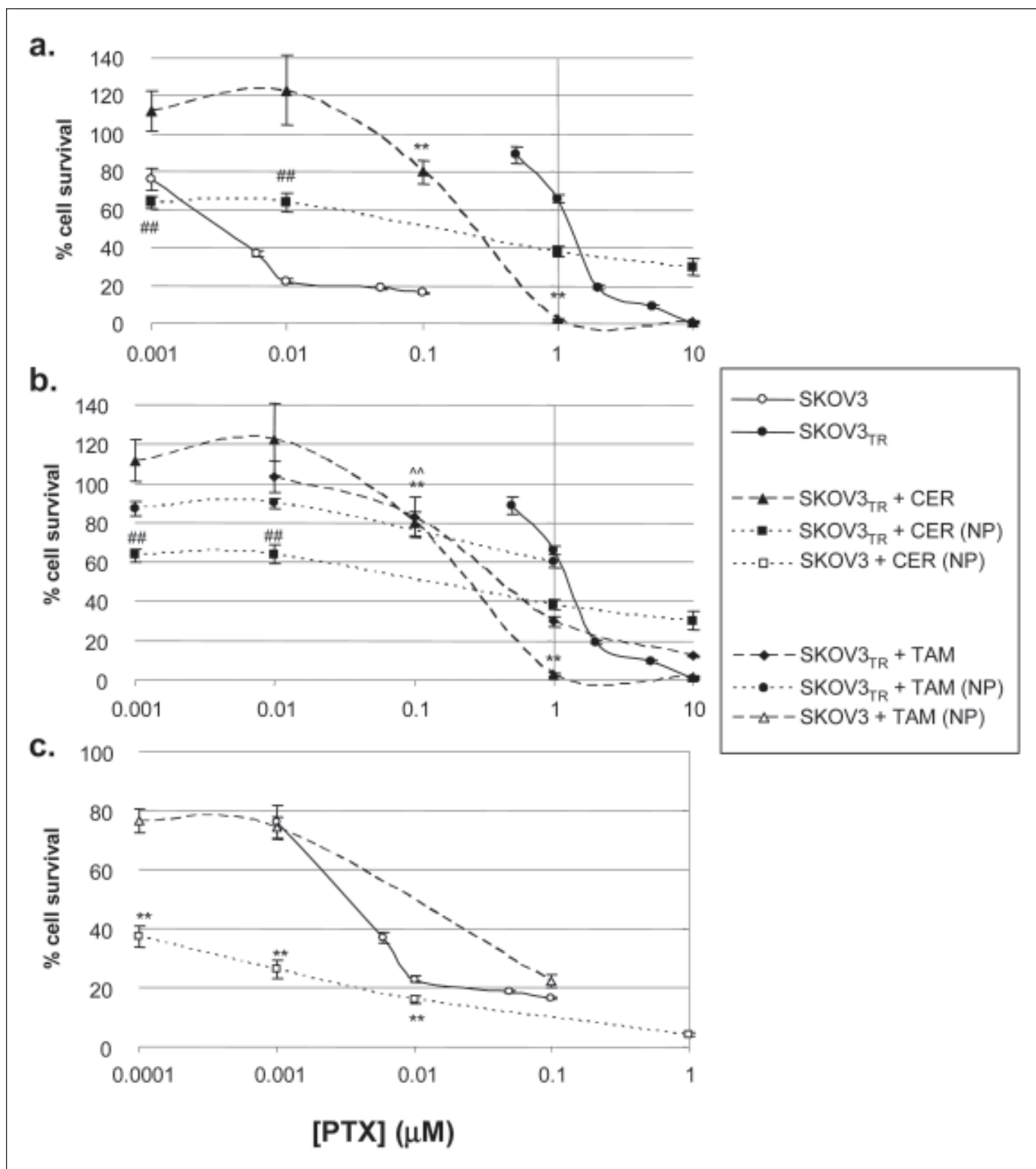


Figure 3. PTX dose response of SKOV3 and SKOV3_{TR} cells with or without a co-therapy as free drug or encapsulated within PEO-PCL nanoparticles (NP). a) comparison of the PTX dose response on SKOV3 and SKOV3_{TR} and the effect of the PTX + CER therapy in solution and in nanoparticles, b) comparison of the PTX dose response in SKOV3_{TR} cells to the PTX + CER therapy and the PTX + TAM therapy, and c) comparison of the PTX dose response in SKOV3 cells and the effect of the PTX + CER and the PTX + TAM therapies; ** indicates a statistically significant difference (p < 0.001) between treatment with PTX alone and PTX + CER within the same cell type, ^^ indicates a statistically significant difference (p < 0.001) between treatment with PTX alone and PTX + TAM within the same cell type, and ## indicates a statistically significant difference (p < 0.001) between treatment with a co-therapy in solution and in nanoparticles (n = 8 samples/group).

same 1 μM dose of PTX alongside 10 μM CER eradicates nearly the entire population ($2.7 \pm 0.5\%$ survival). It is important to note that this dose of CER in itself is not cytotoxic, purposefully chosen to investigate whether this co-therapy acts synergistically rather than additive. However, the combination therapy did not possess the power to revert the MDR chemosensitivity back to the drug-sensitive nature - e.g., a 0.01 μM dose of PTX alongside CER did not result in any cell death - while that same dose of PTX on the SKOV3 cells resulted in a mere $22.7 \pm 1.1\%$ survival. This is likely due to the remains of other mechanisms of MDR in the cells. Since it is known that the SKOV3_{TR} cells over-express P-glycoprotein in addition to GCS, and since it is well known that PTX is a substrate for P-glycoprotein efflux, it was of interest to examine whether the former mechanism of MDR could be overcome by this therapy as well. Although nanoparticle encapsulation is mainly for the *in-vivo* benefit of enhanced tumor drug-delivery, it was of interest to see whether nanoparticle drug delivery could lead to intracellular drug delivery, thereby evading the P-glycoprotein efflux machinery, a phenomenon that has been described by several groups.¹⁵⁻¹⁷

Dose-response studies on the SKOV3_{TR} cells interestingly revealed that the combination of CER modulation and nanoparticle delivery did in fact revert chemoresistance even further, as predicted, as seen in Figure 3a. Hereby the 0.01 μM dose of PTX alongside CER that did not revert chemoresistance when delivered as free drugs (solution), resulted in an eradication of nearly half the MDR population ($64.0 \pm 5.0\%$ survival) when delivered to the cells encapsulated within nanoparticles. To verify that this phenomenon indeed occurred due to enhanced intracellular retention of the P-glycoprotein substrate PTX, intracellular levels of drug following solution or nanoparticle delivery were quantitated through the presence of a ³H label on the PTX.

Figure 4 reveals precisely what was expected, mainly that intracellular retention of PTX in the SKOV3_{TR} cells following administration of the un-encapsulated drug was only about half of the amount that retained in the drug sensitive SKOV3 cells, likely explained by the presence of P-glycoprotein-mediated drug efflux in the SKOV3_{TR} cells. However, when the same dose was delivered to the SKOV3_{TR} cells encapsulated in nanoparticles, a significantly greater amount of the dose retained intracellularly. Since this phenomenon was not present in the drug-sensitive SKOV3 cells, which lack P-glycoprotein, the data indeed suggests that the enhanced chemosensitization seen with the nanoparticle-mediated PTX + CER treatment could be due to a modulation of both apoptotic signaling as well as P-glycoprotein drug efflux. However, nanoparticle therapy lacked this profile at higher doses of PTX, and in fact resulted in less chemosensitization at these doses than the solution co-therapy. This is likely explained by the fact that the internalization of nanoparticles into cells is a saturable process, whereby the cell saturation limit of these particles had been reached at these higher doses of PTX. Nonetheless, it is the objective to obtain cell-kill at lower therapeutic doses of PTX in the MDR phenotype, thus

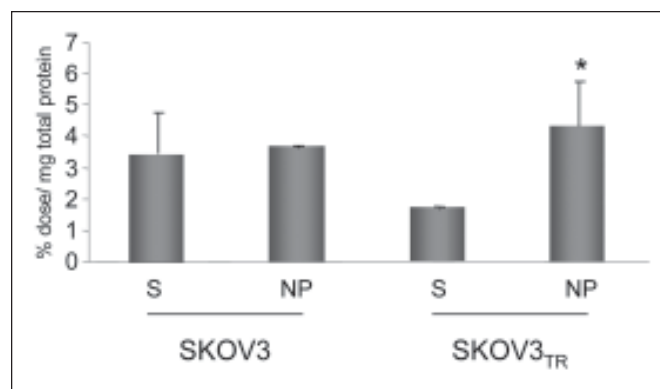


Figure 4. Intracellular paclitaxel (PTX) accumulation in SKOV3 and SKOV3_{TR} cells treated with 0.1 μM PTX containing a ³H-PTX label (1.5 $\mu\text{Ci}/\text{mg}$ drug) in solution (S) or in PEO-PCL nanoparticles (NP) after a six hour treatment period; * indicates a statistically significant difference ($p < 0.05$) between S and NP drug accumulation ($n = 3$ samples/group).

the effect of this therapy at lower doses of PTX is of greater importance.

Since the CER co-therapy aimed to re-instate the defects in apoptotic signaling, it was of importance to verify that chemo-sensitization of MDR by this combination approach is indeed due to a restoration of apoptotic signaling. To verify this, the SKOV3_{TR} cells were stained for apoptotic activity at 12 hours after treatment initiation, by staining with green-fluorescent YO-PRO-1TM and red-fluorescent Propidium Iodide (PI). Blue-fluorescent Hoechst staining was included as an internal control for cell count. Apoptotic activity was measured by laser scanning cytometry with simultaneous fluorescence microscopy. Data supports the notion that the PTX and CER combination therapy indeed restores apoptotic signaling to overcome MDR, as seen by the 2-fold increase in apoptotic activity in cells treated with the combination therapy compared with treatment with PTX alone - Figure 5.

Modulating MDR through a feedback of exogenous CER to re-instate the CER signal has been shown to be successful. However, it was of interest to see if the same phenomenon occurs when GCS is blocked in the MDR cell line, therein preventing endogenous CER from undergoing metabolism to glucosylceramide. The drug tamoxifen (TAM) has been reported to inhibit GCS;¹⁸ therefore, it was speculated that a combination therapy of PTX with TAM would produce the same chemosensitization profile as the PTX + CER therapy. Figure 3b shows that this combination of PTX + TAM indeed also chemosensitized the MDR cell type, to a similar degree as the PTX + CER co-treatment. And like the PTX + CER treatment, the PTX + TAM treatment was similarly enhanced by nanoparticle delivery, e.g., while the co-therapy in solution at a 0.001 μM PTX dose did not produce any cell kill, the co-therapy delivered in nanoparticles at this dose resulted in slight cell kill ($87.2 \pm 3.8\%$ viability). However, like the PTX + CER nanoparticle therapy, the PTX + TAM nanoparticle therapy also exhibited saturation of cell internalization at the higher doses of PTX.

Unlike prior generations of MDR modulation strategies,

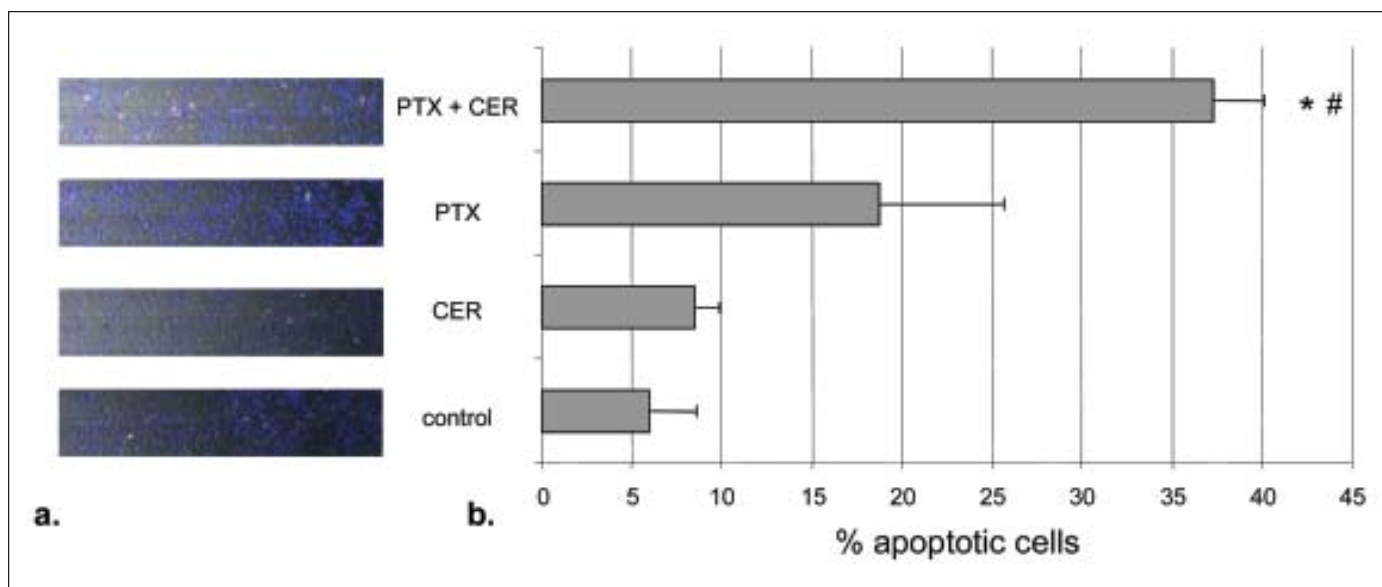


Figure 5. Apoptosis in SKOV3_{TR} at 12 hours following treatment with C₆-ceramide (CER), paclitaxel (PTX), and combination of PTX + CER. Control refers to cells that did not receive any treatment. a) Fluorescence microscopy images of SKOV3_{TR} cells a 12 hour treatment, stained positive for apoptosis with green-fluorescence YO-PRO-1™ and red-fluorescence PI, along a blue-fluorescent Hoechst counter-stain for cell count. b) Percent apoptotic cells per treatment. * indicates a statistically significant difference ($p < 0.05$) from control, # indicates a statistically significant difference ($p < 0.05$) from PTX and CER ($n = 3$ samples/group).

therapeutically aimed at mechanisms particular to the MDR phenotype, modulation of the apoptotic signal also could enhance chemosensitization of drug sensitive cells. Figure 3c illustrates how the PTX + CER nanoparticle therapy greatly improves chemosensitization of the SKOV3 cells, as seen by a left-shift of the dose-response curve. Although the SKOV3 cells benefit from the addition of exogenous CER to induce cytotoxicity, it was not expected that they would respond to the PTX + TAM co-therapy since the drug-sensitive cells do not suffer from an overexpression of GCS. And indeed, the results verify that the PTX + TAM nanoparticle therapy did not enhance chemosensitivity in the SKOV3 cells. These results indicate not only the importance of GCS-mediated CER metabolism and apoptotic modulation as an important contributor to the MDR phenotype, but moreover, they reveal the success of an apoptosis modulation strategy to not only revert MDR in cancer, but also chemosensitize non-MDR cancer types.

Conclusions

Since the development of MDR in cancer greatly hinders success of chemotherapeutic approaches, thereby limiting patient prognosis and survival, therapeutic strategies to circumvent MDR are greatly needed. Although prior MDR modulation attempts seemed promising, clinical success of these therapies remains inconclusive, fueling the drive toward alternate approaches to overcome MDR.

The modulation of apoptotic signaling has emerged as an important mechanism in the MDR phenotype, offering promising potential as a therapeutic target to overcome MDR. However, since MDR is most likely due to multiple mechanisms within the cancer cell, a multifunctional therapeutic strategy that simultaneously overcomes multiple mecha-

nisms of MDR would be beneficial. In this work, we have developed a therapeutic strategy that would deliver a combination therapy of PTX and CER packaged within polymeric nanoparticles to overcome MDR by a multifunctional approach. While exogenous CER administration aimed to restore the defects in apoptotic signaling, nanoparticle delivery of the combination therapy aimed to not only improve systemic drug delivery to the tumor site, but also deliver the drugs intracellularly, thereby evading P-glycoprotein mediated drug efflux. The data support the ability of this novel therapeutic to chemosensitize MDR cancer by this multi-prong approach. And unlike prior MDR modulation strategies, this novel therapeutic has been shown to enhance chemosensitization of non-MDR (drug sensitive) cancer cells as well. Together, these results support the promising clinical potential for this therapy to overcome MDR in cancer.

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