Modulation of Intracellular Ceramide using Polymeric Nanoparticles to Overcome Multidrug Resistance in Tumor Cells

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ABSTRACT

The development of multidrug resistance (MDR) in many tumor types is a major barrier to successful anti-cancer therapy. Among the mechanisms that lead to such chemoresistance is inhibition of apoptotic signaling in MDR cancer cells through glycosylation of the apoptotic mediator ceramide. Glucosylceramide, subsequently cannot propagate the apoptotic signal to result in cell death, thereby rendering these cancer cells drug resistant. The purpose of this study was to co-administer exogenous ceramide with a chemotherapeutic (paclitaxel), encapsulated in polymeric nanoparticles, to investigate whether MDR could be reverted by this multifunctional anticancer therapy.

Keywords: PEO-PCL nanoparticles, MDR, apoptosis, ceramide

1. INTRODUCTION

A major clinical obstacle in cancer therapy is the development of resistance to a multitude of chemotherapeutic agents, a phenomenon termed as multidrug resistance (MDR). The development of drug resistance in a small subset of tumor cells is believed to be the cause for tumor survival despite invasive chemotherapy [1]. Drug resistance can be classified as either inherent or acquired, where inherent drug resistance is caused by genetic predisposition, while acquired drug resistance is developed in response to prolonged antineoplastic treatment. Cancer cells can acquire multidrug resistance through several molecular mechanisms, and often more than one mechanism may be responsible for the MDR phenotype. Causes for multi-drug resistance include overexpression of membrane spanning ATP-dependant drug efflux pumps from the ABC transporter family, modifications in glutathione-S-transferase activity, alterations in DNA repair mechanisms, and modification of apoptotic signaling [1].

Another major barrier to successful anti-tumor therapy is the challenge to deliver the required therapeutic concentration at 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 amount of the dose at the target site, which minimizes the amount of therapeutic that accumulates at non-specific targets. Poly(ε-caprolactone) (PCL) nanoparticles are useful drug delivery carriers for such tumor targeted delivery [2]. The alkyl structure of the polymer efficiently encapsulates hydrophobic compounds, and allows for slow degradation of the particle for extended release of the drug. Surface modification of the colloidal carrier with a poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO-PEO) triblock copolymer improves solubility of the nanoparticle in the aqueous environment of the body, while also repelling plasma proteins, decreasing immune activation, and increasing circulation time of the nanoparticles in the plasma. The nanoparticles then accumulate preferentially at the tumor site due to the enhanced permeability and retention effect; circulation of the drug within the polymeric nanoparticles increases the probability that the nanoparticles reach the tumor vasculature, where they easily extravasate through the fenestrations in tumor capillaries into the tumor mass to deposit the drug at the intended target site. By this mechanism, concentrations of drug inside the tumor cell can be 10-100 fold higher than when administering free drug [3].

Paclitaxel (PTX) is an anti-tumor chemotherapeutic derived from the bark of the Pacific yew tree (Taxus brevifolia), that is widely used in the treatment of solid tumors, particularly of the breast and ovaries [4]. PTX exerts its cytotoxicity by inducing tubulin polymerization resulting in unstable microtubules, which interferes with mitotic spindle function and ultimately arrests cells in the G2/M phase of mitosis [5]. Tumor cells exposed to PTX treatment then, as a result, undergo programmed cell death (apoptosis), which is essentially how PTX exerts its anti-tumor effect. Although it is understood that cell cycle arrest results in activation of the apoptotic signaling cascade, recent studies suggest that PTX therapy may also cause direct accumulation of endogenous ceramide, a lipid with function as a cellular second messenger in apoptosis [6].

Ceramide (CER) is derived intracellularly by hydrolysis of the lipid sphingomyelin, but it can also be
produced de novo through N-acylation of sphinganine. Accumulation of endogenous CER, produced by either hydrolysis or de novo formation, is known to result in response to several stimuli, such as growth factor deprivation, pro-inflammatory signals, exposure to increased temperature and radiation, and other stressors such as chemotherapeutics and related cytotoxic agents [7]. Among such stimuli, paclitaxel has been shown to elevate intracellular CER levels in breast tumor cells [6]. Accumulation of intracellular CER is implicated in the cellular responses to stress, such as apoptosis and cell cycle arrest, where CER functions as a second messenger in the signaling cascade that initiates these responses. 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]. While active CER is accrued by sphingomyelin hydrolysis or de novo production from sphinganine in the cell, CER can subsequently be further metabolized by glycosylation to yield glucosylceramide, a non-toxic form of CER that is not implicated in the initiation of apoptosis [9]. The enzyme glucosylceramide synthase (GCS), also known as CER glucosyltransferase or UDP-glucose-N-acylsphingosine D-glucosyltransferase, is responsible for this inactivation of CER [9]. Several MDR tumor cell lines have exhibited elevated levels of non-cytotoxic glucosylceramide and corresponding elevated levels of GCS [9], and clinical studies have suggested that glucosylceramide levels are elevated in tumor specimens of breast cancer and melanomas that were poorly responsive to chemotherapy [10]. These findings not only suggest the importance of CER in the mediation of the cytotoxic response to anti-tumor chemotherapeutics, but they also suggest that inhibition of apoptotic signaling, by inactivation of endogenous ceramide, may be an important mechanism whereby tumors develop multi-drug resistance.

The purpose of this study was to overcome the barriers of MDR tumor therapy by 1) elevation of intracellular CER levels as a potential co-treatment to reverse paclitaxel resistance in an ovarian tumor cell line (SKOV3) and 2) to administer the treatment in poly(ethylene oxide)-modified poly(ε-caprolactone) (PEO-PCL) nanoparticles to maximize drug delivery to the tumor site and thus enhance the anti-tumor response. Exogenous C6-CER was co-administered with paclitaxel in solution or in PEO-PCL nanoparticles, and its therapeutic potential was compared to that with similar delivery of paclitaxel or CER alone.

2. EXPERIMENTAL METHODS

2.1 Nanoparticle preparation and characterization

PEO-PCL nanoparticles were prepared by controlled solvent displacement using an acetone-water system, and loaded with 10% w/w PTX or 20% w/w C6-ceramide (CER). The nanoparticles were formed by dissolving the drug/polymer mixture in acetone, followed by gentle addition of the polymer-drug solution to distilled water under rapid magnetic stirring. Following evaporation of the organic solvent, nanoparticles were collected by centrifugation, washed in distilled water, and lyophilized for storage. Nanoparticle preparations were subsequently subjected to size and zeta-potential measurements using a Brookhaven 90Plus analyzer. For visual nanoparticle tracking, identical batches of PEO-PCL nanoparticles were prepared, but loaded instead with 0.1% w/w rhodamine-PTX or 0.1% w/w NBD-CER.

2.2 In-vitro cytotoxicity studies

The wildtype (drug sensitive (DS)) human ovarian cancer cell line SKOV3 was maintained in culture alongside an SKOV3TR subculture that was selected for multidrug resistance in the presence of increasing concentrations of PTX. For cytotoxicity studies, 96-well plates were prepared with each cell type seeded at 5000 cells/well. The DS and MDR cells were subjected to dose-response studies against PTX, CER and PTX combined with CER, delivered as free drugs in solution or delivered in PEO-PCL nanoparticles. All studies were performed alongside a negative control (cell growth medium), a positive control (polyethylenimine, PEI), and proper vehicle controls. Since PTX is a cell-cycle dependant chemotherapeutic, all treatments were left to proceed undisturbed for 6 days, allowing ample time for all cells to enter mitosis. Resulting cell death/viability was measured by the MTS (formazan) assay.

2.3 Apoptotic activity assay

To determine the potential for CER co-administration to revert apoptotic signaling in the MDR cells, apoptotic activity was measured. A commercially available kit (Vybrant #7, Invitrogen) was used to fluorescently stain apoptotic cells with green fluorescent Yo-Pro and late apoptotic or necrotic cells with propidium iodide in addition to Yo-Pro, distinguishing them from live cells which remained unstained. Blue fluorescent Hoechst-33342 was used as an internal control for cell count. SKOV3 and SKOV3TR cells were plated in 96-well optical quality plates at a density of 2x10^4 cells/well. Both cell populations were subjected to treatment with PTX alone, CER alone, PTX in combination with CER, or vehicle (negative control). Treatment proceeded undisturbed for 24 hours to induce apoptosis, at which point cells were stained for apoptotic activity followed by in-situ cytometric analysis of live cells using the iCys® microplate cytometry platform (Compucyte Corp., Cambridge, MA) that combines laser scanning cytometry with fluorescent microscopy. The iCys® platform allows for simultaneous excitation and absorption of the three dyes for quantitative cell sorting and fluorescent microscopy in one scan. Yo-Pro and PI were excited at 488 nm by an argon laser and
absorbed at 515-545 nm and 600-635 nm respectively, while Hoechst was excited at 405 nm by a diode laser and absorbed at 445-485 nm. Each sample scan was repeated 4 times, and all treatments were run in triplicate.

3. RESULTS AND DISCUSSION

Controlled solvent displacement produced reproducible PEO-PCL nanoparticles with a mean diameter of 211.6 ± 1.8 nm and surface charge of -31.09 ± 1.53, characteristic of PEO-PCL. Trafficking studies with the rhodamine-PTX and NBD-CER labeled nanoparticles visualized how these nanoparticles are engulfed and trafficked towards the center of the cell to deposit their load (Figure 1). This intracellular drug deposit helps avoid P-glycoprotein mediated drug efflux characteristic of MDR, which potentially explains the enhanced cell kill effect that nanoparticle-mediated drug delivery has over delivery of free drugs in solution by diffusion that will be shown in Figure 2.

Dose-response studies determined the IC50 of drug sensitive (SKOV3) cells to PTX in solution to be around 5 nM (data not shown), therefore the low-dose (safe-dose) PTX treatment was set at 1 nM while the high-dose (toxic dose) PTX treatment was set at 100 nM. Figure 2 indicates that at 1 nM PTX, 76.03 ± 5.92% of cells survived, while at 100 nM PTX only 16.37 ± 0.41% of cells survived, verifying that 100 nM is a highly cytotoxic dose for the wild-type cells. On the other hand, treatment of the SKOV3TR cells with 100 nM PTX resulted in 100.00 ± 5.78% viability, verifying that the MDR cells are indeed significantly resistant to PTX. Treatment with 1 µM of PTX resulted in 65.65 ± 2.16% viability, setting this dose as the experimental cytotoxic dose for the resistant cells. Co-treatment of the SKOV3TR cells with 20 µM C6-CER in addition to 1 µM PTX resulted in a significant increase in cell death (2.69 ± 0.51% viability) compared with the PTX treatment alone (p<0.001), while addition of CER did not significantly enhance cytotoxicity at the 100 nM dose of PTX. Treatment with CER alone at 20 µM did not significantly enhance cytotoxicity either (91.18 ± 7.61% viability). Addition of 10 µM C6-CER to 100 nM PTX dose to the SKOV3 (drug sensitive) cells also resulted in enhanced cell death (7.38 ± 1.25% viability compared with

![Image](image1.png)

Figure 1 – Intracellular accumulation of PEO-PCL nanoparticles loaded with rhodamine-PTX (red) and NBD-CER (green) in SKOV3 cells at 6 hours incubation. The left panel shows the combined fluorescent signal of PTX and CER loaded nanoparticles, while the right panel shows an overlay of fluorescence on the DIC image of the cells.

16.37 ± 0.41% with PTX alone), while, similar to the PTX resistant cells, CER co-treatment did not significantly enhance cytotoxicity at the low PTX dose (70.84 ± 2.62% viability with the co-treatment). CER treatment alone (10 µM dose) did, however, cause slight cytotoxicity in the PTX sensitive cells (75.08 ± 7.86%, p<0.05).

Although the purpose of drug encapsulation within nanoparticles is for the *in-vivo* benefits of prolonged circulation and tumor-specific drug accumulation, encapsulation of PTX and CER within nanoparticles showed therapeutic benefit over treatment of drug in solution *in-vitro* as well. Treatment with a 10-fold lower concentration of PTX (10 nM) with CER in nanoparticles showed a greater percentage of cell death than treatment with 100 nM PTX with CER (63.98 ± 4.90% viability with nanoparticles vs. 79.78 ± 6.18% viability with treatments in solution) (figure 2). Moreover, encapsulation of the drugs in nanoparticles sensitized the PTX resistant cells to both the high (1 µM) PTX dose as well as the low (10 nM) PTX dose when co-treated with CER (110.58 ± 3.84% viability lowered to 63.98 ± 4.90% viability for the low dose, and 85.09 ± 6.30% viability lowered to 38.15 ± 2.58% viability for the high dose, p<0.001 for both cases). Recall that co-treatment of the drugs in solution resulted only in enhanced cytotoxicity at the high (toxic) dose without affecting cytotoxicity at the low (safe) dose. The same results were obtained with similar treatments in the PTX sensitive cells.

To determine if the enhanced cytotoxicity in response to CER/PTX co-treatment results from the hypothesized restoration of apoptotic signaling, we stained the SKOV3 and SKOV3TR cells for apoptotic activity at the same conditions set forth in the cytotoxicity studies (Figure 2). Apoptotic activity was measured 24 hours following treatment initiation by microplate cytometry and confirmed by simultaneous fluorescent microscopy. As shown in Figure 3b, the co-treatment of PTX/CER results in a two-fold increase in the amount of apoptotic activity in the MDR cells over PTX treatment alone, where the concentration of PTX in any of the treatments is merely 10 nM (recall that at 10 nM PTX, 100% of the MDR cells survived). Additionally, exogenous administration of CER with PTX also doubles the amount of apoptotic activity in the drug sensitive SKOV3 cells, although, this result is
expected given the decrease in cell viability with PTX/CER co-treatment shown in the cytotoxicity studies (Figure 2). It is possible, and suggested by the data, that the enhanced apoptotic activity and cell death with the co-treatment in the SKOV3 cells is due to an additive effect of individual PTX and CER cytotoxicities, since there does not appear to be a significant increase in cell death unless the concentration of CER used has a significant cell-kill effect on its own. However, in the MDR cells, there is significant enhancement of cell death when combining concentrations of PTX and CER that individually do not result in significant cell-kill. For example, in the top-right panel of figure 2, treatment with CER alone does not produce significant cell death (91.2 ± 7.6% cell viability) while treatment with PTX alone (1 µM) still results in 65.6 ± 2.2% cell viability. However, when combined, treatment at these same concentrations causes nearly 100% cell death (2.7 ± 0.5% cell viability), a phenomenon that, in the MDR cells, is not likely explained by additive PTX and CER cytotoxicities. Thus, it is assumed from the results that a feedback of exogenous CER indeed restores the blocked apoptotic signal initiated by PTX cytotoxicity in the MDR cells, although further studies are needed to confirm this.

4. CONCLUSION

Altogether, a therapeutic strategy that co-administers PTX with CER, delivered in polymeric nanoparticles appears to greatly re-sensitize drug resistant ovarian tumor cells to chemotherapy. The results demonstrate the great potential for clinical use of this therapeutic strategy to overcome MDR.

5. ACKNOWLEDGEMENTS

This study was supported by NIH grants CA-095522 and CA-119617.

REFERENCES