3D Cancer Cell Spheroids as a Model For Investigating Co-Delivery of Paclitaxel and Curcumin by Targeted Mixed Micelles

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ABSTRACT SUMMARY
In this study, co-delivery of paclitaxel (PCL) and curcumin (CUR) by transferrin (TF)-targeted PEG-PE-based mixed micelles was investigated using multidrug resistant (MDR) human ovarian cancer cell monolayers, spheroids and in vivo tumors. Co-delivery of PCL and CUR resulted in synergistic cytotoxicity against MDR cells. Results suggest that micelles simultaneously delivering PCL and CUR, or micelles loaded with only PCL but targeted with TF result in same activity, both in vitro and in vivo.

INTRODUCTION
Multicellular 3D cancer cell culture (spheroids) resembles in vivo tumors in terms of shape, cell morphology, growth kinetics, gene expression and drug response. The outer-region cells of a spheroid are actively proliferating, while inner-region cells are in non-proliferative state. Their 3D structure consisting of extensive amount of ECMs causes a complex interaction with cell-to-cell and microenvironment. At the same time, these characteristics cause very limited drug penetration into deeper parts of both the spheroids and in vivo tumors thus, decreased response to anticancer drugs [1]. Resistance to anticancer drugs, known as multidrug resistance (MDR), is dependent on both biochemical and physical obstructions such as overexpressed efflux pumps (i.e. P-gp), upregulated pathways (i.e. NF-kB and PI3K) and limited penetration of drugs.

Most of the cancer research involving the drug resistance mainly focuses on the biochemical mechanisms, while the limitations of drug penetration into the tumor tissues in vivo also plays an underestimated but crucial role. With this in mind, PEG-PE-based mixed micelle formulations co-loaded with PCL and a potent NF-kB inhibitor, CUR, were prepared with TF as the targeting ligand. We hypothesized that: (i) micellar encapsulation and delivery would enhance the spheroid/tumor penetration and toxicity of the drugs, (ii) adding TF as the targeting ligand would increase the penetration of the micellar systems into the spheroids/tumors, and (iii) simultaneous delivery of CUR and PCL would result in synergistic cytotoxicity. Human resistant ovarian carcinoma cell monolayers, spheroids, and in vivo tumors were used to evaluate targeted and co-loaded micellar nanocarrier systems.

EXPERIMENTAL METHODS
Multicellular NCI-ADR-RES human MDR ovarian carcinoma cell spheroids were prepared by liquid overlay method using agar-coated (1.5% w/v in serum free DMEM) 96-well plates as described previously [2]. PCL and/or CUR drug-loaded mixed micelles based on 1,2-Diesteraryl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG–PE) were prepared by the thin film hydration method. PCL and/or CUR at various weight % of the polymer were added to PEG–PE and vitamin E (89:11 molar ratio) solution in chloroform. To obtain targeted micelles, TF was reacted with pNP-PEG3400-PE and incorporated into the micelles at 1% mole ratio to PEG–PE.

Cellular association of TF-targeted or non-targeted micelles with spheroids and monolayers was assessed by flow cytometry using fluorescently labeled (rhodamine-PE) micelles. Distribution of micelles in spheroids was analyzed by confocal microscopy. The in vitro cytotoxicity of different micellar formulations was investigated against NCI-ADR-RES monolayers and spheroids using CellTiter Blue® and CellTiter-Glo® after 72 hours of treatment.

Similarities between the spheroid model and in vivo model of human resistant ovarian cancer were investigated in nude mice bearing SK-OV-3TR tumors. Targeted and non-modified micelles containing CUR and PCL were used to investigate the tumor inhibition efficacy of formulations. PCL and CUR doses were 10 mg/kg and 25 mg/kg, respectively.

RESULTS AND DISCUSSION
CUR and PCL, two hydrophobic drug, successfully co-loaded into mixed micelles at concentrations of 1216.7±82.3 µg/mL and 482.5±37.3 µg/mL, respectively. All formulations have the hydrodynamic diameter ranging within 15–20 nm with negative zeta potentials.

TF-targeting did not result in significantly higher cellular association with monolayers after 4 hours. In spheroids, confocal imaging demonstrated significantly deep penetration of micelles after the same incubation time (Figure 1B and 1C). However, to achieve significantly higher cellular internalization with targeted micelles, additional 2 hours were needed. Even after 6 hours, the fluorescence intensity increase in spheroids was still significantly lower compared to monolayers, which indicates the limited micelle penetration into the spheroid structure (Figure 1A).

In monolayers, both free drugs, PCL and CUR, showed significantly less cytotoxicity than micellar formulations. TF-modification did not cause significantly higher cytotoxicity after 72 hours of treatment, regardless of co-loading (Figure 2). While varying the concentration
of PCL in the co-loaded micelles and keeping the CUR concentration constant at 5 µM, the combination delivery resulted in significantly higher and synergistic toxicity compared to PCL micelles alone at 0.8 µM PCL concentration, while CUR itself at 5 µM concentration did not show significant toxicity.

In spheroids, micellar encapsulation resulted in significant decrease in cell viability after 4 hours. (Figure 3). When CUR was combined with PCL, we did notice a significant decrease in cell viability due to the synergistic effect. But TF-targeting of the co-loaded micelles did not result in significant increase in the cytotoxicity compared to non-targeted co-loaded formulations.

The main objective of the in vivo study was to investigate the TF-targeting effect on the co-loaded micelles. The combination treatment has exhibited tumor growth inhibition, suggesting that combining the anticancer effect of PCL with resistance reversal effect of CUR is a promising approach, even at low PCL dose at 10 mg/kg (Figure 4). On the other hand, TF modification of the co-loaded micelles did not caused significant decrease in the tumor volumes, similar to the results obtained with spheroids.

Figure 3. NCI-ADR-RES spheroids cell viability after 72 hours of treatment (mean±SD, n=3).

Figure 4. Tumor inhibition studies with various micellar formulations. n ≥5 per group and all values are expressed as mean±SEM, * P<0.01, ** P<0.001.

CONCLUSION
We have found in spheroid model, that significantly higher doses of the drugs need to be used compared the monolayers, to obtain same levels of cytotoxicity due to the altered cellular pathways, overexpression of P-gp and limited penetration into the 3D structure. Co-delivery of CUR at non-cytotoxic concentrations with PCL, synergistically increased PCL cytotoxicity due to sensitizing effect of CUR. TF-targeting provided clear advantages over non-modified micelles in terms of better penetration and cellular association, and significantly increased the cytotoxicity of single agent-loaded micelles. However, when PCL and CUR co-loaded micelles were used, TF-targeting did not further increase the cytotoxicity, both in spheroids and in vivo tumors. When CUR and PCL co-loaded micelles were used, further TF-targeting becomes unnecessary for this active compound combination, which eliminates additional steps and improves the overall stability of the micelle system. The effect of co-loading and TF-targeting resulted in similar responses in vivo and in the 3D cell culture, which suggests that the spheroid model can be used as an intermediate model for evaluation of co-delivery of anticancer compounds by targeted micelles.

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REFERENCES