Enhanced Cancer Cell Cytotoxicity of Dual Ligand-Targeted Doxorubicin-Loaded Liposomes

Shravan Kumar Sriraman and Vladimir P. Torchilin

Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, Boston, MA 02115
sriraman.s@husky.neu.edu

ABSTRACT SUMMARY
Many cell-surface receptors like the transferrin (TfR) and folic acid receptors (FR) are over-expressed in many cancer cells. This receptor expression increases as the tumor progresses. With this in mind, polyethylene glycol (PEG)-coated long-circulating liposomes targeted with both, transferrin and folic acid were loaded with doxorubicin (Dox) and evaluated on human cervical cancer cells (HeLa). The dual-ligand liposomes (DL) demonstrated a 7-fold increase in cell association as compared to the single-ligand liposomes (SL) as well as an enhanced cytotoxic effect in vitro even at low drug concentrations.

INTRODUCTION
Cancer, one of the leading causes of human deaths worldwide, originates from the uncontrolled cell growth of normal cells. The fact that it stems from an organism’s own cells makes it harder to selectively treat it with chemotherapy. Traditional chemotherapeutic agents kill cancer cells as well as a majority of rapidly dividing healthy cells in the body leading to disastrous side-effects. Although the liposomal delivery of chemotherapeutic agents has served to alleviate many of such problems by the passive accumulation at tumor sites via the enhanced permeability and retention (EPR) effect, there is still ample room for improvement.

Ligand-targeted liposomes allow for the increased accumulation of the liposomal cargo at the target site thus resulting in improved therapeutic outcomes and reduced drug-associated toxicity. It has been found that cancer cells over-express different types of cell-surface receptors like TfR and FR when compared to normal cells in order to fuel their aggressive growth. Nanoparticles targeting these receptors have also been shown to evade the action of drug efflux pumps typical of resistant tumor types. This led us to hypothesize that the simultaneous targeting of these receptors using an optimized combination of ligands could enhance therapeutic outcomes in a variety of regular and drug-resistant tumors.

EXPERIMENTAL METHODS
Liposomes were prepared by the thin film hydration method followed by extrusion through polycarbonate membranes using a hand-held extruder. Transferrin and folic acid were conjugated to the distal ends of the PEG blocks in PEG-PE conjugates and post-inserted into the liposomes along with PEG2000-DSPE by overnight incubation at 37°C. All experiments were carried out using HeLa cells. For cell association studies, liposomes labeled with rhodamine were incubated with the cells for 4 hours (0.1mg/mL lipid concentration) and subsequently analyzed by confocal microscopy and flow cytometry. For all in vitro cytotoxicity experiments, liposomes were loaded with Dox by the pH-gradient method at a Dox to lipid mole ratio of 1:5. Cells were incubated with the dox-loaded formulations for 4 hours and washed with media. Their viability was measured after 48 hours using the Promega cytotoxicity assay. N=3 for all experiments unless otherwise mentioned.

RESULTS AND DISCUSSION
Flow cytometry demonstrated a 7-fold increase in cell association of the DL compared to any SL formulations (see Figure 1). This association and subsequent internalization was further confirmed using confocal laser scanning microscopy (data not shown).
Figure 1. Mean fluorescent intensity of rhodamine-labeled liposomes (L) targeted with either folic acid (F), transferrin (T) or both (F+T) analyzed by flow cytometry.

This increased cellular association of the DL over the SL was also successfully translated into an enhanced cytotoxic effect in vitro as is seen in Figure 2. The DL significantly reduced the IC50 values compared to the SL and LD. This allows to use much lower drug concentrations thereby preventing Dox-associated non-specific toxicity.

CONCLUSION
The simultaneous use of two targeting ligands, such as folate and transferrin, have been shown to be an effective strategy to deliver liposomal drugs specifically and enhance their cytotoxic effects on cancer cells in vitro over SL. This selectivity allows to use lower drug concentrations thereby reducing the potential off-target drug toxicity.

REFERENCES

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