ABSTRACT SUMMARY

Bortezomib (Velcade), a proteasome inhibitor, was encapsulated in two types of liposomal nanocarriers viz. cationic or ceramide in order to enhance its intracellular delivery and apoptosis induction. Cellular viability assay and apoptosis assay was conducted in related cell lines. By delivering bortezomib using liposomal nanocarriers cell death and apoptosis was increased in a time and dose dependent manner in comparison to bortezomib alone.

INTRODUCTION

Multiple myeloma (MM) is a malignancy of terminally differentiated B-lymphocytes (plasma cells). The cancerous cells overwhelm the bone marrow and are associated with anemia and bone pain. The standard of care for MM is bortezomib, a proteasome inhibitor that is capable of inducing myeloma cell apoptosis. Bortezomib must avoid lysosomal degradation while being delivered to its target, the proteasome located inside the cell. Targeted drug delivery to B cells using particulate drug carriers has been proven to be effective in the past and could potentially reduce the side effects of bortezomib, such as peripheral neuropathy. Liposomes may enhance delivery as they are the “carrier of choice” to prevent such degradation. Two formulations of liposomes, cationic (DOTAP) and ceramide, were developed to enhance intracellular delivery of bortezomib. Cationic liposomes were chosen due to previous studies showing enhanced apoptotic induction. This effect was correlated to generation of reactive oxygen radicals from macrophages because of higher cell uptake of cationic liposomes by macrophages through electrostatic interaction between the cationic charge of the liposomes and the anionic charge of the cells (Takano et al. 2003). Ceramide liposomes were chosen due to the fact that they are known to be important intracellular signaling molecules that mediate apoptosis (Shabbits and Mayer 2003). Ceramide lipids are recognized to participate in signal transduction cascades by activating serine/threonine kinases and by stimulating protein phosphatases. Ceramides have also been shown to exert direct effects on mitochondria and is thought to mediate apoptosis via channel or pore formation in the mitochondrial membranes. To investigate which formulation had better synergistic apoptotic effects, the changes in cellular metabolic activity were assessed on three different cell lines with the Alamar Blue assay. Cell death via the apoptotic pathway was assessed by flow cytometry.

To better understand the consequences of bortezomib on bone cells, three different cell lines were tested: an osteoblast mouse calvaria cell line (MC3T3), a pre-osteoclast mouse cell line (RAW264.7), and an osteosarcoma human cell line (U2OS).

EXPERIMENTAL METHODS

1. Preparation and characterization of liposomes

Liposomes were prepared by dry film evaporation technique, characterized in terms of particle size, zeta potential, and loading efficiency. The ceramide liposomes consisted of C6-Pyridinium-Ceramide (Avanti Polar Lipids, AL), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EPC-35, Avanti Polar Lipids, AL), cholesterol, and bortezomib (LC Laboratory, MA) and the cationic liposomes contained only 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, Avanti Polar Lipids, AL) and bortezomib. Both were separately dissolved in methanol and chloroform. The solvent was evaporated using a Buchi rotary evaporator, dried under nitrogen gas, and re-dissolved in HEPES 10mM EDTA 1mM buffer pH 7.5. The liposomes were extruded through a series of 100/100 nm filters and analyzed using a zetasizer, Nano-ZS by Malvern Instruments. The percentage of loading was then determined by HPLC analysis.

2. Cell viability assay using Alamar Blue

Cells were grown in either DMEM/F12 (U2OS), αMEM (MC3T3), or DMEM (RAW264.7) medium with 10% FBS, and incubated at 37°C. Metabolic activity was assessed by plating 1x10^4 cells/well in 96 well plates and incubated overnight in a humidified chamber with 5% CO2. Cells were treated with 0.064 nM to 1000 nM concentrations of either bortezomib or bortezomib encapsulated in ceramide or cationic liposomes for 24 or 48 hours. Alamar Blue (Invitrogen, NY) was added to the plates and read using a Beckman Coulter DTX 800 multi-mode plate reader with Ex 535/Em 595 filters. Statistical analysis was performed using Graphpad Prism (Version 5.0). A nonlinear regression analysis was used to determine IC50 values.

3. Apoptosis assay

Flow cytometry using FITC Annexin V Apoptosis Detection Kit with Propidium Iodide (Biolegend, CA) was performed on RAW cells. Cell plates were plated with 1x10^4 cells. After 24 hours of incubation, cells were treated with either bortezomib alone or bortezomib encapsulated in ceramide liposomes, at 0nM, 1nM, 10nM, or 100nM. Flow cytometry using an annexin V/propidium iodide staining assay kit was performed with analysis on a FACSCalibur (BD Biosciences) as per manufacturers recommendation 24 hours post treatment. Statistical analysis, a student ‘t’ test, was performed using Graphpad Prism (Version 5.0).

RESULTS AND DISCUSSION

Both liposomal formulations were prepared multiple times and measured for particle size, polydispersity index,
Treatment with cationic bortezomib liposomes resulted in significantly reduced metabolic activity in MC3T3 cells, RAW 264.7 cells, and U2OS cells compared to bortezomib alone at 24 h and 48 h, respectively (all p<0.01). These results were quite distinct from ceramide bortezomib liposomes which were found to have higher pIC50 values compared to bortezomib alone at 24 and 48 h of exposure in all three cell lines, but only the pre-osteoclast cell line (RAW 264.7) were found to be statistically significant at both 24 h and 48 h (p<0.01 and p<0.05, respectively), Table 2.

Table 2. The pIC 50 (-log IC50) values of bortezomib and either Ceramide or cationic bortezomib liposomes on different cell lines was calculated using Graphpad prism (Version 5.0). Higher values indicate exponentially greater potency. (* p < 0.05; ** p < 0.01)

In the apoptosis assay using florescent-activated cell sorting (FACS), on RAW cells pretreated with bortezomib alone and encapsulated in ceramide, the latter resulted in higher number of cells undergoing apoptosis (lower right (LR) quadrant) at the 1 nM concentration (p<0.01) and higher number of cells undergoing both cell death and apoptosis (upper right (UR) quadrant) at the 10 nM concentration (p<0.05). Both results were statistically significant, Figure 1.

The possibility of targeting therapy for MM by encapsulating bortezomib into liposomes could lead to treatments with increased efficacy and decreased side effects such as peripheral neuropathy known to occur with this chemotherapy. It is worth noting that ceramide liposomes themselves can induce apoptosis in some cell lines and may have resulted in synergistic apoptotic activity with the bortezomib.