The influence of drug loading on caveolin mediated intracellular internalization of Doxorubicin Nanomicelles

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ABSTRACT SUMMARY

Despite the large progress made in the field of anticancer nanomedicine, the influence of the physicochemical characteristics of different Nanoconstructs on the internalization and cytotoxicity of specific cancer cells remains to be fully understood. The aim of this study was to establish the factors influencing the efficiency of the doxorubicin loaded styrene maleic acid (DOX-SMA) micelle against different breast and prostate cell lines in vitro. A significant difference in cytotoxicity relative to micelle loading was observed and correlated with the expression level of caveolin. DOX-SMA micelles co-localized with caveolin in PC3 cells that was disrupted by treatment with genistein. In conclusion micelle loading and cellular expression of caveolin determines the cytotoxicity of micelles in breast and prostate cell lines.

INTRODUCTION

The discovery of EPR effect offered considerable momentum to the field of anticancer Nanomedicine, however, over the past 30 years, only nine nanomedicines have been approved (1). A possible explanation is the lack of knowledge about the physicochemical characteristics of nanomedicine necessary for an effective treatment of specific tumors. Doxorubicin was encapsulated into the polymer SMA to form micelles with a wt/wt loading range of 4.4% to 28.4%. The cytotoxicity of SMA-DOX against prostate cancer cell lines (DU145 and PC3 cells) and breast cancer cell lines (MDA-MB-231 and MCF7 cells). The cytotoxicity of the micelles relative to the loading varied significantly relative to the cell type being treated. This was determined to be a consequence the relative level of caveolin expression impacting the quantity of endocytosed doxorubicin.

EXPERIMENTAL METHODS

SMA micelles were prepared by adjusting anhydrous SMA to pH 5.0 and adding Ethyl dimethyl aminopropyl carbodiimide and doxorubicin to the solution. The pH was raised to 11 to trigger the micelle formation. Then, pH was adjusted to 7.4 and the solution filtered and lyophilized. All measurements for size and zeta potential were carried out using the Malvern ZEN3600 Zetasizer nano series. Releas was determined by dissolving micelles in PBS (pH 7.4) at a concentration of 1 mg/ml. Briefly, 1.5 ml of solution was placed in a 1,000 kDa cutoff membrane in a 50 ml flacon tube. 15 ml of PBS was placed outside of the dialysis tube and the tubes were incubated at 37°C, 65 RPM for 96 hours. At the indicated times 2 ml of the solution outside the bag was removed and the absorbance read at 478 nm before being placed back in the tube.

Cytotoxicity was determined by seeding MDA-MB-231, PC3, DU145 and MCF7 cells in 96 well-plates and incubating for 24 h at 37°C in 5% CO2 before treating with a range of concentrations from 0 to 10 µM of doxorubicin equivalent. The cells were incubated for 72 h and fixed using trichloracetic acid. Cytotoxicity was measured by determining the cell number using the sulforhodamine B assay.

In order to visualize endocytic vesicles and endocytosed doxorubicin micelles, PC3 cells were treated with 200 µM genistein, an inhibitor of caveolin dependent endocytosis, for 1 hour. Cells were then treated with 25 µM doxorubicin loaded micelles and incubated for 4 hours. Cells were fixed, washed and permeabilised. Cells were incubated with antibodies against caveolin at 4°C overnight, washed and incubated with FITC conjugated secondary antibody for 1 hour at room temperature. Cells were stained with DAPI before being mounted on slides.

RESULTS AND DISCUSSION

SMA encapsulation of doxorubicin yielded final drug loadings of 4.4%, 14.5% and 28.4%. The loading of the micelles had a significant influence on solubility s with lower loading resulting in increased solubility.

![Figure 1: Release rate of doxorubicin micelles in pH 7.4 PBS.](image)

The size of the 4.44%, 14.5% and 28.4% micelles was established to be 10.68 nm, 12.4 nm and 14.59 nm respectively. The release of the 4.44%, 14.5% and 28.4% micelles following 96 hours incubation was 50%, 25% and 10% respectively (figure 1). The cytotoxicity of doxorubicin micelles was examined in DU145, PC3, MCF-7 and MDA-MB-231 cells as these cells represent commonly diagnosed cancers. Relative to
free doxorubicin, the toxicity of micellar doxorubicin was reduced in all cell lines, likely because free doxorubicin enters the cell in a diffusion limited manner while micellar doxorubicin must be endocytosed. The observed cytotoxicity showed distinct and different patterns in all four cell lines (figure 2). MDA-MB-231 showed negligible dependence of cytotoxicity on loading at all concentrations examined while PC3 cells showed little difference at higher doxorubicin concentrations. MCF7 and DU145 cells showed a clear delineation between the cytotoxicity observed with different loadings however MCF-7 cells showed a low maximal response.

The dependence of cytotoxicity on cell type indicated that a particular endocytosis process may be important for modulating this effect. To examine this possibility, the relative cellular expression of proteins involved in different stages or routes of endocytosis, caveolin, clathrin and early endosome antigen 1 (EEA1), were examined using Western blot. The Western blot showed little difference between clathrin and EEA1 expression in the cell lines. Conversely, the difference in caveolin expression between the cell lines was substantial. MDA-MB-231 cells showed very high expression of caveolin, PC3 cells showed median expression, DU145 cells showed low expression and MCF-7 cells showed undetectable caveolin expression.

This expression pattern suggests that the lack of differential cytotoxicity observed with MDA-MB-231 cells may be due to a high endocytic rate resulting in maximal toxicity at all concentrations. DU145 cells show a lower expression of caveolin in comparison to MDA-MB-231 cells, the amount of doxorubicin endocytosed becomes important for the observed cytotoxicity and largely influenced by the loading of each micelle.

In order to determine if caveolin and doxorubicin micelles co-localized, immunocytochemistry was employed. Doxorubicin micelles treatment showed co-localization with caveolin. Treatment for 1 hour with genistein abolished co-localisation of caveolin and doxorubicin, suggesting that the differential cytotoxicity may be a result of the ability of the cell to endocytose the SMA. For example in MCF-7 and DU145 cells, which show low levels of caveolin expression, the differential cytotoxicity observed with different loaded micelles is clear while in MDA-MB-231 cells which show high expression of caveolin there is little difference in cytotoxicity.

CONCLUSION
In conclusion, caveolin mediated endocytosis appears to be the primary mechanism by which cells endocytose SMA micelles and caveolin expression has a significant impact on the cytotoxicity observed following treatment with SMA micelles. Consideration of micellar loading and caveolin expression should be made in choosing the tumor types that may be responsive to SMA micelles.

REFERENCES