Development of a tubulin fractionation assay for the evaluation of "on-target" activity of tubulin targeting agents in clinical PBMC samples

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Introduction

- A method for confirming on-target activity of tubulin binding agents using human clinical samples was developed.
- Tubulin polymer concentration in peripheral blood mononuclear cells (PBMCs) was used as a surrogate biomarker of drug activity for the novel vascular disruption agent, BNC105.
- BNC105 is a tubulin polymerization inhibitor that selectively disrupts tumor vasculature and suppresses growth in a broad range of solid tumor models.
- BNC105P is the phosphorylated parent compound that is rapidly converted to the active agent, BNC105, following intravenous administration.
- The method was applied to demonstrate on-target activity of BNC105 in phase I clinical trial PBMC samples.

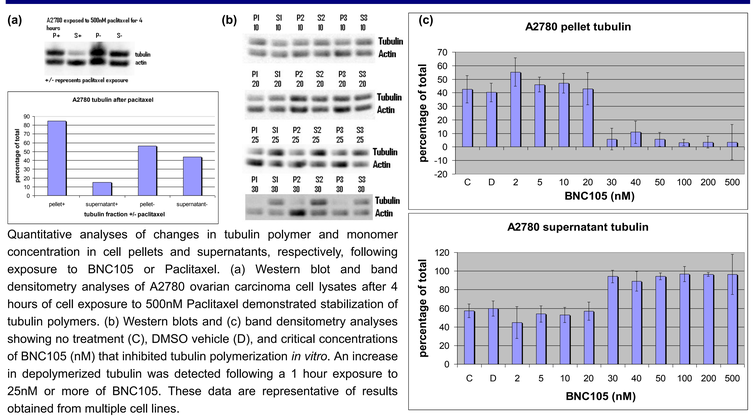
Overview of the experimental procedure used to measure the effects of tubulin targeting agents

PATIENT PBMCs
↓
MAINTAIN MICROTUBULE POLYMER
↓
RUPTURE CELL MEMBRANE
↓
HIGH SPEED CENTRIFUGATION
↓
SEPARATE TUBULIN FRACTIONS
↓
WESTERN IMMUNOBLOT FOR TUBULIN
↓
QUANTITATE TUBULIN FRACTIONS

This method was used to demonstrate on-target activity of the tubulin depolymerising agent, BNC105. Cancer cell lines and human volunteer PBMCs were used as surrogate cell populations to develop and optimize the assay. The method capitalizes on mass differences between polymerized and monomeric tubulin to separate each fraction into cell pellets (polymerized tubulin) or cell supernatants (monomeric tubulin) by centrifugation. A lysis buffer specifically designed to maintain tubulin polymers and facilitate separation of polymerized tubulin fractions from monomeric tubulin fractions was used to disrupt cell membranes immediately prior to centrifugation. Each fraction was subjected to SDS-PAGE and Western blotting using anti β1-tubulin and anti β-actin antibodies to detect tubulin and actin protein bands. ImageJ software (NIH, Bethesda, USA) was used to quantify protein band density for tubulin and actin in cell pellets and cell supernatants. The numeric representation of tubulin band density was divided by the numeric representation of actin band density to normalize for loading differences between samples. Normalized values were converted to percentages of total tubulin in the combined cell pellet and supernatant, and plotted on bar graphs to facilitate comparisons between drug-treated and untreated cells.

A method was developed to measure the effects of tubulin-targeting drugs by exploiting mass differences between polymerized and monomeric tubulin

Tubulin polymer changes in cell lines following exposure to BNC105 or Paclitaxel



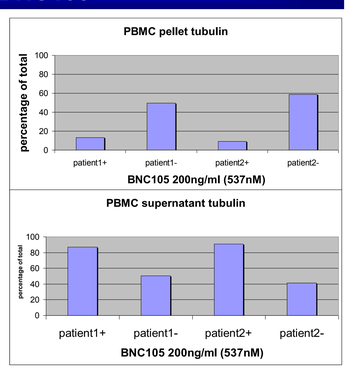
Quantitative analyses of changes in tubulin polymer and monomer concentration in cell pellets and supernatants, respectively, following exposure to BNC105 or Paclitaxel. (a) Western blot and band densitometry analyses of A2780 ovarian carcinoma cell lysates after 4 hours of cell exposure to 500nM Paclitaxel demonstrated stabilization of tubulin polymers. (b) Western blots and (c) band densitometry analyses showing no treatment (C), DMSO vehicle (D), and critical concentrations of BNC105 (nM) that inhibited tubulin polymerization *in vitro*. An increase in depolymerized tubulin was detected following a 1 hour exposure to 25nM or more of BNC105. These data are representative of results obtained from multiple cell lines.

A clear tubulin depolymerization effect was observed following exposure to BNC105 in cancer cell lines *in vitro*

Tubulin depolymerization in human PBMCs following exposure to BNC105

Blood was collected from eight volunteers into BD vacutainer CPT™ (Becton Dickinson) blood collection tubes containing sodium citrate as an anticoagulant. BNC105 was added to whole blood samples at 537nM, and samples were rocked at room temperature for one hour prior to centrifugation to isolate PBMCs according to the manufacturers protocol. Tubulin fractions were separated from BNC105-treated and untreated PBMCs and analyzed by Western immunoblotting and band densitometry.

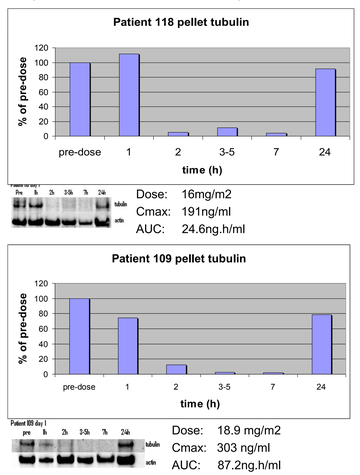
The concentration of polymerized tubulin in cell pellets from samples that had been exposed to BNC105 decreased, while concomitantly the concentration of monomeric tubulin in the corresponding cell lysates increased. This shift was not observed in control samples. These results demonstrated that tubulin in PBMCs was affected by BNC105 in a similar manner to the tubulin in cancer cell lines, and indicated that PBMC tubulin from clinical samples may also be affected following intravenous administration of BNC105P. Tubulin from patient PBMCs was subsequently investigated as a potential surrogate biomarker for BNC105 on-target activity.



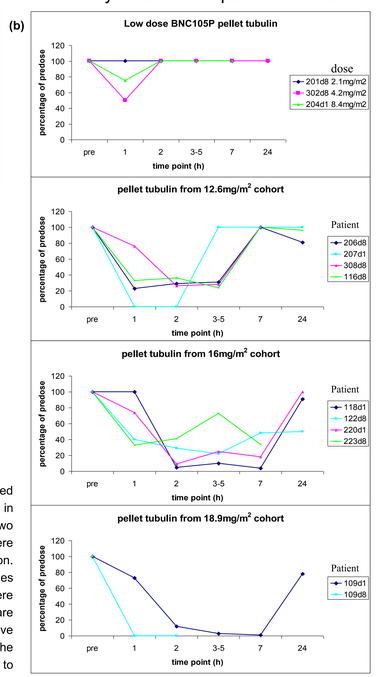
Tubulin polymer reduction was observed in human PBMCs following exposure to BNC105 *in vitro*

Tubulin depolymerization in phase I patient PBMCs following intravenous administration of BNC105P

Experimental data from two patients



Summary of data from patient cohorts



The effects of BNC105 on PBMC tubulin polymers could be detected following the IV administration of doses at and above 12.6mg/m^2 in phase I clinical trial patients. (a) Representative results from two patients illustrate how the analyses of patient PBMC samples were performed over a 24 hour time period following drug administration. Densitometry values were converted to percentages of pre-dose values and represented on the y axes, while blood collection time points were represented on the x axes. (b) Results from each patient cohort are represented on line graphs as percentages of tubulin polymer relative to the pre-dose tubulin polymer concentration, with 100% set as the maximum value. These data demonstrate a clear dose-response to BNC105, with a dramatic reduction in polymerized tubulin following drug administration that returned to pre-dose levels within 24 hours.

The intravenous administration of BNC105P in phase I clinical trial patients resulted in a quantifiable reduction in patient PBMC tubulin polymer concentration

Summary and Conclusion

- A method was developed to evaluate the on-target activity of tubulin binding agents using tubulin from cell lines and PBMCs as a surrogate biomarker of drug activity.
- Analyses of cell lines demonstrated quantifiable changes in tubulin polymer concentration in response to tubulin targeting agents.
- Clinical samples from a phase I study of the novel tubulin targeting agent, BNC105, were analysed following drug administration, and a clear dose-response was demonstrated.