**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 disrupting agent, BNC105.
- BNC105 is a tubulin polymerization inhibitor that selectively disrupts tumour vasculature and suppresses growth in a broad range of solid tumour cell lines.
- 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 clearance activity of the tubulin depolymerising agent, BNC105. Cancer cell lines and human volunteer PBMCs were used as surrogates to population cultures to develop and optimise the assay. The method capitalizes on the fact that the concentration of polymerized tubulin in cell pellets from samples that had been exposed to BNC105 decreased, while concurrently 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 manner similar to the tubulin in cancer cell lines, and indicated that BNC105 could be used to detect on-target activity in PBMCs.

PBMC samples were collected from clinical trial volunteer PBMCs treated and untreated cells. Tubulin from PBMCs was separated into polymerized and monomeric tubulin fractions. Western blotting using anti-tubulin antibody was used to quantify protein band density for tubulin and actin in cellular pellets and supernatants. ImageJ software (NIH, Bethesda, USA) was used to quantify protein band density for tubulin and actin in cellular pellets and supernatants. Each fraction was subjected to SDS-PAGE and Western blotting using anti-tubulin antibody to disrupt cell membranes immediately prior to centrifugation to isolate PBMCs according to the manufacturers protocol. Tubulin fractions were separated from PBMCs, and quantified by densitometry analyses of A2780 ovarian carcinoma cell lysates after 4 hours of cell exposure to 500nM Paclitaxel demonstrated stabilization of polymerized tubulin. Western blot analysis showed no treatment (A), DAPI vehicle (B), and drug concentrations of BNC105 (C) that inhibited tubulin polymerization in A2780 cells. An increase in depolymerized tubulin was detected following 1 hour exposure to 250nM 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. PBMCs were isolated from blood samples at 0, 1, 2, 3, 5, 7, 24, and 48 hours after drug administration. Tubulin from PBMCs was separated into polymerized and monomeric tubulin fractions and quantified by Western immunoblotting and densitometry analyses of A2780 ovarian carcinoma cell lysates after 4 hours of cell exposure to 500nM Paclitaxel demonstrated stabilization of polymerized tubulin. Western blot analysis showed no treatment (A), DAPI vehicle (B), and drug concentrations of BNC105 (C) that inhibited tubulin polymerization in A2780 cells. An increase in depolymerized tubulin was detected following 1 hour exposure to 250nM of BNC105. These data are representative of results obtained from multiple cell lines.

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

**Summary and Conclusion**

A method was developed to evaluate the “on-target” activity of tubulin binding agents using PBMC samples and PBMCs as a surrogate biomarker of drug activity.

- Analyses of cell lines demonstrated quantitative 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.

The intravenous administration of BNC105 in phase I clinical trial patients resulted in a quantifiable reduction in patient PBMC tubulin polymer concentration.

**Experimental data from two patients**

**Summary of data from patient cohorts**

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

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