Harnessing the tumor adaptive response to hypoxia to identify novel combinations of the vascular disrupting agent BNC105 with targeted therapeutics

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BACKGROUND. BNC105 is a Vascular Disrupting Agent (VDA) that exerts anti-cancer activity through the selective shut-down of tumor blood vessels. It selectively disrupts the relatively chaotic and fast growing vasculature within a tumor resulting in cessation of oxygen and nutrients delivered to the tumor. A single dose of BNC105 causes a high degree of hypoxia and necrosis within 24hrs post treatment in rodent tumor models. Disruption of tumor vasculature following treatment with BNC105 was also observed in cancer patients (Rischin et al. 2011). The degree of hypoxia within the tumor varies as the cells proximal to the tumor periphery draw on normal vasculature which is not disrupted by BNC105 activity. Tumor recovery from the hypoxic stress occurs by day 2 post-treatment as the remaining cells within the tumor microenvironment adapt to the altered conditions. We have conducted immunohistochemical analysis to identify the molecular basis driving this tumor recovery. Using the mouse renal cancer orthotopic tumor model RENCA, BNC105 causes significant vascular shutdown within the tumor followed by activation of proteins involved in tumor adaptive responses to hypoxia. Similar observations were recorded from BNC105 treated mice bearing tumors of the human renal cancer cell lines Caki-1 (VHL wild type) and A-498 (VHL mutant). These observations led us to investigate the potential therapeutic benefit of combining BNC105 with agents inhibiting the function of proteins being upregulated as a result of BNC105 induced tumor hypoxia. The increase in intra-tumor hypoxia caused by BNC105 treatment can be leveraged to gain greater therapeutic benefit by targeting tumor adaptive responses.

EFFECTS OF BNC105 ON RENAL TUMOR VASCULATURE

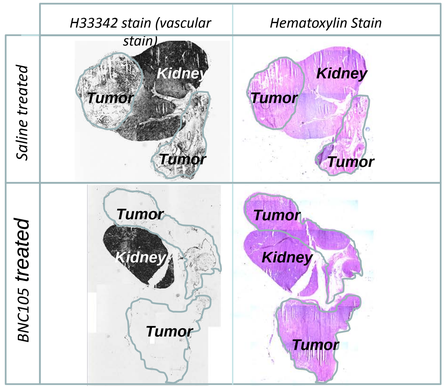


Figure 1: A single iv dose of BNC105 (32 mg/kg) causes disruption of vasculature in RENCA cell orthotopic tumors with no effect on the vasculature of the kidney. Vascular function is visualised through iv injection of H33342, 1 min prior to animal euthanasia. Slides were post-stained with Hematoxylin to differentiate normal kidney and tumour. Maximum tumor vascular disruption was seen as early as 1hr post treatment, with necrosis reaching maximum levels 24hrs post treatment. Tumor recovery from the effects of BNC105 is evident by 2 days post treatment. RENCA cells - (mouse renal adenocarcinoma; ATCC CRL-2947, VHL wildtype).

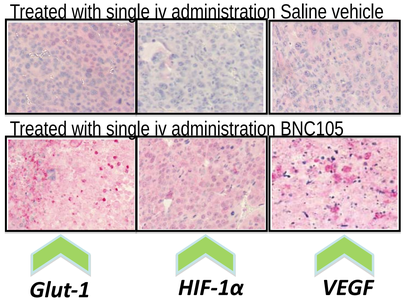
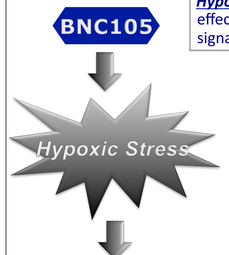


Figure 2: Tumor vascular disruption induced by BNC105 leads in the upregulation of markers associated with tumor response to hypoxia. Formalin fixed paraffin embedded RENCA tumor sections from Balb/c mice collected 24 hours after a single dose of 32mg/kg BNC105 were examined for expression of markers previously shown to be involved in tumor adaptive responses to hypoxia. Our data show up-regulation of the hypoxia markers Glut-1 (Acris AP06144PU-N), Hif1α (Santa Cruz sc10790) and VEGF (Abcam ab46154). (Fast Red detection on Hematoxylin). These markers have been shown to drive tumor survival and induction of angiogenesis.

- ***↑ Glut-1, increases glucose transport across cell membrane (Warburg effect)***
- ***↑ HIF1α, gateway to a cascade of hypoxic molecular responses***
- ***↑ VEGF, recruits vasculature to microenvironment***

BNC105 INDUCES ACTIVATION OF THE mTOR PATHWAY

Hypothesis: Renal tumors recover from the hypoxic stress caused by the vascular disruption effect of BNC105 through upregulation of mTOR signaling. Concurrent blockade of mTOR signaling reduces ability of renal tumors to recover from hypoxic stress.



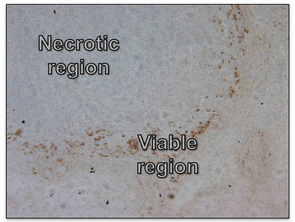


Figure 3: Increased mTOR phosphorylation occurs in tumor zones surviving the necrotic effects of BNC105. Frozen sections from Caki-1 (human renal clear cell carcinoma; ATCC HTB-46, VHL wildtype) xenografts in Balb/c nude mice collected 24 hrs after a single iv dose BNC105 (32mg/kg) demonstrates necrotic and viable tumor regions. Phosphorylation of mTOR (Cell signalling #2855) (DAB detection on Hematoxylin) was clearly seen in the viable tumor region. This may drive tumor recovery from the effects of BNC105.

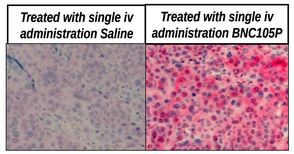


Figure 4: BNC105 induces increase in 4EBP1 phosphorylation. Formalin fixed paraffin embedded RENCA tumor sections from Balb/c mice collected 24 hrs after a single iv dose BNC105 (32mg/kg) were stained for expression of 4EBP1 phosphorylation (Cell signalling #2855). (Fast Red detection on Hematoxylin)

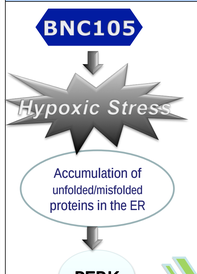
Table 1: A498 (human renal carcinoma; ATCC HTB-44, VHL mutant) xenografts were treated with Rapamycin 1mg/kg ip (Day 1, 4, 8 & 11) and BNC105 32mg/kg iv (Day 4 & 11). The Rapamycin + BNC105 combination treatment resulted in statistically significant ($p<0.05$) tumor growth inhibition compared to the monotherapies showing that the two compounds have a complimentary mechanism of action.

Treatment	Day 5	Day 8	Day 12
	TGI%		
BNC105	10	12	14
Rapamycin	19	18	27
Combination	42	47	51

- *BNC105 induced hypoxia upregulates the mTOR survival pathway*
- *Concurrent treatment with rapamycin (mTOR inhibitor) sensitizes the A498 cell line to the effects of BNC105, with the BNC105+rapamycin treatment group showing greater tumor growth inhibition*

BNC105 INDUCES ACTIVATION OF THE UNFOLDED PROTEIN RESPONSE

Hypothesis: BNC105 induced hypoxia induces accumulation of misfolded proteins and ER stress. Tumor cells recover from these effects through upregulation of PERK, phosphorylation of eIF2a and activation of the Unfolded Protein Response (UPR). BNC105 treatment and concurrent inhibition of the proteasome with Bortezomib will disrupt UPR, increase ER stress and result in increased and more sustained tumor necrosis.



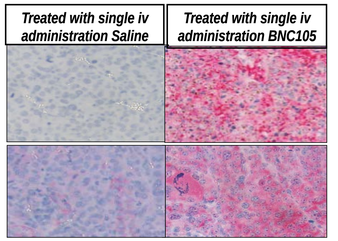


Figure 5: Formalin fixed paraffin embedded RENCA orthotopic tumor sections from Balb/c mice collected 24 hrs after a single dose iv 32mg/kg BNC105 demonstrates upregulation of unfolded protein response markers PERK (Cell signalling #5683) and phosphorylation of eIF2α (Cell Signalling #5324). (Fast Red detection on Hematoxylin)

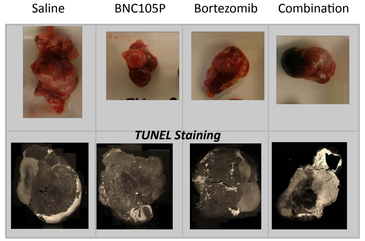


Figure 6: RENCA orthotopic tumour bearing kidneys from Balb/c mice were photographed 4 hours after dosing BNC105 on Day 9 of treatment (Bortezomib 0.5mg/kg iv Days 1, 5 & 8, BNC105 32mg/kg iv Days 2 and 9). Formalin fixed and paraffin embedded sections were stained using a TUNEL assay (Roche). BNC105 induced hypoxia leads to detectable tumor necrosis 24hrs after BNC105 monotherapy but not earlier. Concurrent treatment with Bortezomib induces necrosis at the 4 hr timepoint.

- *Treatment of RENCA tumors with BNC105 + Bortezomib resulted in increased tumor necrosis compared to animals treated with Bortezomib or BNC105 alone. This suggests that the tumor is more susceptible to BNC105 induced hypoxia when in combination with Bortezomib*

- **Regions of tumours adapt to the hypoxic shock caused by BNC105 treatment through adaptive survival pathways such as the mTOR pathway and the Unfolded Protein Response**
- **Targeting these hypoxic adaptive survival pathways has the potential to increase the therapeutic benefit offered by BNC105.**

Reference: Rischin D, Bibby DC, Chong G, Kremmidiotis G, Leske AF, Matthews CA, Wong SS, Rosen MA, Desai J. Clinical, pharmacodynamic, and pharmacokinetic evaluation of BNC105P: a phase I trial of a novel vascular disrupting agent and inhibitor of cancer cell proliferation. Clinical Cancer Research. 2011; 17(15):5152-60