Induction of tumor hypoxia synergises with inhibitors of tumor metabolic and angiogenic adaptive responses to suppress tumor growth and enhance host survival

Daniel J. Inglis, Tina C. Lavranos, Donna M. Beaumont, Annabell F. Leske, Chloe K. Brown, Gabriel Kremmidiotis

Bionomics Ltd, Australia

Molecular Targets and Cancer Therapeutics 2013
Abstract #B67

Introduction

BNC105 is a compound that exerts an anti-cancer action through selective destruction of tumor blood vessels. A single IV dose of BNC105 causes a very high degree of tumor hypoxia leading to >95% necrosis in rodent models. Despite the dramatic tumor necrosis, tumor recovery becomes evident by day 2 following BNC105 treatment. We conducted immunohistochemical, *in vitro* and *in vivo* studies to identify the cellular and molecular basis driving tumor recovery from the significant vascular destruction caused by BNC105. Renal cancer cell lines treated with BNC105 exhibited increased expression of HIFα, VEGFA and increased phosphorylation of mTOR and 4EBP1. These observations led us to investigate the potential therapeutic benefit of combining BNC105 with agents inhibiting the signalling pathways corresponding to these proteins.

Tumor Treatment with BNC105

Highly specific vascular disruption

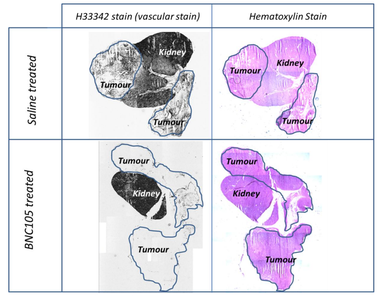

Figure 1: *The perfusion of the vascular stain H33342 into RENCA (mouse renal adenocarcinoma; ATCC CRL-2947, VHL wildtype) orthotopic tumors in balb/c mice demonstrates under fluorescent microscopy the highly selective disruption of tumor vasculature by a single dose of 32mg/kg BNC105P i.v. leaving normal vasculature intact. Slides were post-stained with Hematoxylin to differentiate normal kidney and tumor*

Zonal mTOR upregulation

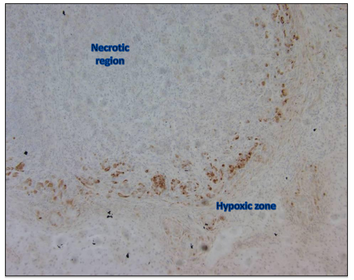

Figure 2*: Frozen section from Caki-1 (human renal clear cell carcinoma; ATCC HTB-46, VHL wildtype) xenografts in balb/c nude mice collected 24 hours after a single dose 32mg/kg BNC105P i.v. demonstrates a necrotic region of the tumor and a hypoxic zone in which phosphorylation of mTOR (Cell signalling #2855) (DAB detection on hematoxylin) drives the recovery of the tumor.*

Adaptive responses activated

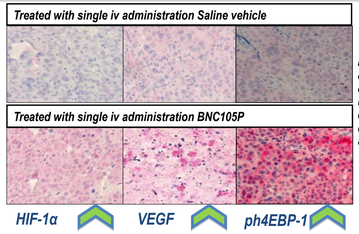

Figure 3: *Formalin fixed paraffin embedded RENCA (ATCC) orthotopic tumor sections from Balb/c mice collected 24 hours after a single dose of 32mg/kg BNC105P i.v. demonstrates upregulation of the hypoxia markers Hif1α (Santa Cruz sc10790) and VEGF (Abcam ab46154). Activation of the mTOR pathway is also evidenced by the phosphorylation of 4EBP1 (Cell Signaling #2855) (Fast Red detection on Hematoxylin).*

We hypothesised that targeting the mTOR pathway and VEGF driven revascularisation of tumors with the mTOR inhibitor Everolimus or pan-VEGFR inhibitor Pazopanib in combination with BNC105 would lead to greater therapeutic benefit.

BNC105 Combination with Everolimus

A panel of renal cancer cell lines, including VHL mutant and VHL wild type, were shown under both normoxic and hypoxic conditions to express high levels of VEGFA. Culturing these cell lines with the mTOR inhibitor Everolimus decreased phosphorylation of p70S6K (Figure 4) and significantly reduced VEGFA expression (Figure 5). These findings demonstrate that Everolimus effectively curtails VEGFA signalling and is appropriate to combine with BNC105 therapy.

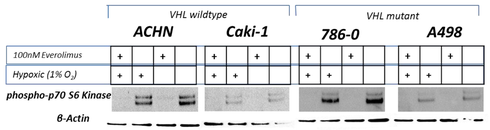

Figure 4: *Human RCC cell lines ACHN (renal cell adenocarcinoma, ATCC CRL-1611), Caki-1, 786-0 (renal cell adenocarcinoma, ATCC CRL-1932) A498 (renal cell carcinoma ATCC HTB-44) were exposed to 100nM Everolimus or vehicle in a normoxic or hypoxic (1% O_2) environment for 6 hours prior to cells being lysed and protein isolated. Protein was quantified using a BCA™ Protein Assay kit (Pierce) and equal amounts resolved using 4%/12% SDS-PAGE (Life Technologies). Blot was probed for phospho-p70 S6 Kinase (Cell Signaling #9234) prior to being striped and probed for β-actin (Cell Signaling #4970). Everolimus strongly inhibits the phosphorylation of p70 S6Kinase via disruption of the mTORC1 complex regardless of hypoxic or VHL mutation.*

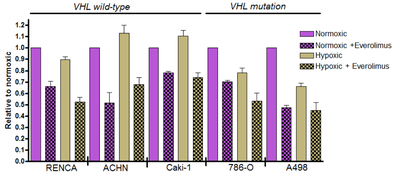

Figure 5: *Human RCC cell lines ACHN, Caki-1, 786-0, A498 and murine RCC cell line RENCA were exposed to 100nM Everolimus or vehicle in a normoxic or hypoxic (1% O_2) environment for 24 hours. Supernatant was collected and total VEGF quantified using a Quantikine® ELISA (Human or mouse specific, R&D Systems). Data was normalised to VEGF production for each cell line in a normoxic environment ± SD. Expression of VEGF is significantly decreased by Everolimus driven mTOR inhibition regardless of hypoxic or VHL mutation status.*

We tested the potential combinatorial benefit of BNC105 + Everolimus *in vivo* using the mouse renal cancer cell line RENCA. Tumor Growth Inhibition (TGI) with Everolimus alone was 46% and BNC105 alone 18%. The BNC105 + Everolimus combination treatment resulted in 73% TGI and was statistically significant compared to the inhibition seen with the monotherapies ($p<0.05$) (Figure 6). Similarly in a human renal carcinoma cell line xenograft (Caki-1), TGI with Everolimus or BNC105 monotherapies was 23% and 25% respectively. In contrast the combination of BNC105+Everolimus produced 46% TGI displaying additive benefit (Figure 7).

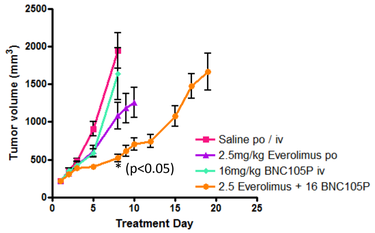

Figure 6: *Female Balb/c nude mice at 6 to 8 weeks old were subcutaneously inoculated with the mouse RENCA renal cancer cell line. Tumors were grown to an average size of 220mm³ and were randomised into groups (n=10) before commencing treatment. Animals were treated with saline (control), 2.5mg/kg Everolimus, or 16mg/kg BNC105P as monotherapies or in combination. Everolimus was dosed p.o. daily and BNC105P i.v. Day 2 and 9 of a 21 day treatment cycle. All animals were checked for health daily and weight and tumor dimensions measured 3-4 times per week. Animals were euthanized upon reaching ethical endpoints based on tumor volume and clinical signs.*

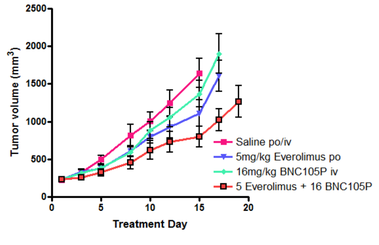

Figure 7: *Female Balb/c nude mice at 6 to 8 weeks old were subcutaneously inoculated with the human Caki-1 renal cancer cell line. Tumors were grown to an average size of 220mm3 and were randomised into groups (n=10) before commencing treatment. Animals were treated with saline (control), 2.5mg/kg Everolimus, or 16mg/kg BNC105P as monotherapies or in combination. Everolimus was dosed p.o. daily and BNC105P i.v. Day 2 and 9 of a 21 day treatment cycle. All animals were checked for health daily and weight and tumor dimensions measured 3 times per week. Animals were euthanized upon reaching ethical endpoints based on tumor volume and clinical signs*

Combination of BNC105 with the mTOR inhibitor Everolimus yields greater anti-tumor efficacy in renal cancer.

BNC105 Combination with Pazopanib *

The potential benefit of combining BNC105 with the pan-VEGFR inhibitor Pazopanib was also investigated. We similarly hypothesised that tumor recovery from the BNC105 induced hypoxia/necrosis insult could be curtailed through inhibition of VEGF receptors. Treatment of RENCA orthotopically implanted tumors with BNC105 alone resulted in 21% TGI and Pazopanib alone 19%. In combination the TGI was 46% (Figure 8A). Furthermore, survival was significantly increased (p=0.0001) than animals treated with monotherapies (Figure 8B).

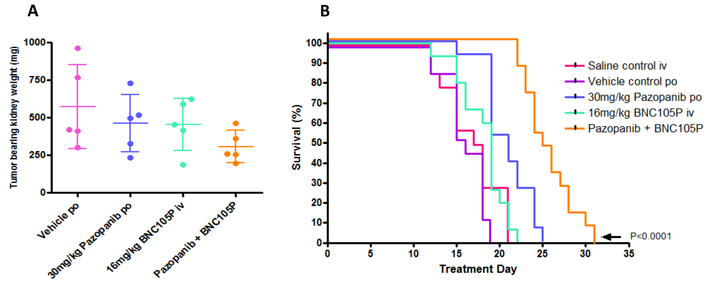

Figure 8: *Female Balb/c mice at 8 weeks were injected orthotopically with murine RENCA renal carcinoma cells under the right kidney capsule. The health and weight of the animals was monitored daily post cell inoculation. On Day 11, 5 animals were euthanized to confirm the establishment of tumors. The remaining animals were then randomised into groups (n=20) and the treatment schedule commenced on Day 12 post inoculation (considered Day 1 of treatment). Animals were treated with saline (control), vehicle (control), 30mg/kg Pazopanib, or 16mg/kg BNC105P as monotherapies or in combination. Pazopanib was dosed p.o. daily and BNC105P i.v. Day 2 and 9 of a 21 day treatment cycle. From each group 5 animals were euthanized at Day 10 of treatment and tumor bearing kidneys weighed (A); the remaining 15 animals per group were monitored daily and euthanized at an ethical endpoint based on body condition and/or clinical signs (B).*

**** Pazopanib was kindly provided by GlaxoSmithKline (USA)***

Combination of BNC105 with the pan-VEGF inhibitor Pazopanib yields greater anti-tumor efficacy and significantly extends host survival in renal cancer.

Summary & Conclusions

- Regions of tumors adapt to the hypoxic shock caused by BNC105 treatment through adaptive survival pathways such as the mTOR pathway and VEGF driven revascularization.
- Specifically targeting and exploiting these hypoxic adaptive survival pathways greatly increases efficacy of renal tumor treatment in combination with BNC105.

- *These data demonstrate that BNC105 can be combined with Everolimus or Pazopanib, to yield greater anti-tumor efficacy in renal cancer.*
- *A randomised Phase II trial evaluating the potential benefit of combining BNC105 with Everolimus in patients with metastatic renal cancer has finished accrual and expected to yield results in the first half of 2014*

A: 31 Dalgleish Street, Thebarton, South Australia, 5031, Australia **E:** dinglis@bionomics.com.au **W:** www.bionomics.com.au