BNC105 is a tubulin targeting agent that is not susceptible to efflux by Pgp transporter expression, and yields additive therapeutic benefit when combined with other anti-cancer agents

Gabriel Kremmidiotis, Donna M Beaumont, Clayton A Matthews, Allison Hall, Jelena Gasic, Renee J Phillips, David C Bibby, Annabell F Leske

Bionomics Limited, 31 Dalgleish Street, Thebarton, South Australia, 5031, Australia

www.bionomics.com.au

Bionomics Limited

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Introduction

The novel anti-cancer agent BNC105 is a benzofuran based tubulin polymerization inhibitor and its prodrug, BNC105P, is a disodium phosphate ester that is rapidly converted into BNC105 *in vivo*. BNC105 exhibits very high anti-tumor efficacy, eliciting a high rate of tumor regressions and tumor burden clearance in many human tumor xenograft models. We have previously shown that BNC105 exerts its anti-tumor activity through a dual mode of action. This agent selectively disrupts the vasculature in tumors, and in addition acts as a direct cytotoxic against cancer cells.

BNC105 has demonstrated single agent efficacy in a number of cell lines and tumor models, including breast, colon, prostate, brain and lung cancer. We have recently evaluated the activity of BNC105 in models of human Head and Neck cancer and investigated its utility in combination with radiation treatment.

Furthermore, we have conducted a number of studies to evaluate the potential synergy of BNC105 with several chemotherapeutic agents currently in clinical use. Our analyses included initial *in vitro* activity investigations in human cancer cell lines followed by assessment of *in vivo* efficacy in animal xenograft models.

One mechanism through which cancer cells develop resistance to treatment is through the upregulation of efflux pumps such as Pgp. Drugs that are not substrates for these efflux pumps have a clear advantage as cancer cells can not use this mechanism to reduce drug efficacy. The potential involvement of Pgp transporters in the efflux of BNC105 was investigated in order to rule out this resistance mechanism.

Evaluation of BNC105 Efficacy in Models of Head and Neck Cancer as a Single Agent or in Combination With Radiotherapy

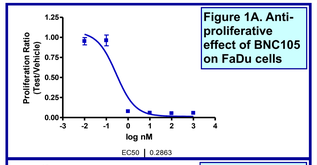

Figure 1A. Anti-proliferative effect of BNC105 on FaDu cells

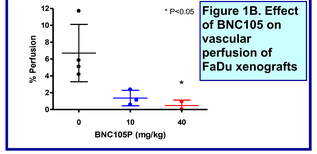

Figure 1B. Effect of BNC105 on vascular perfusion of FaDu xenografts

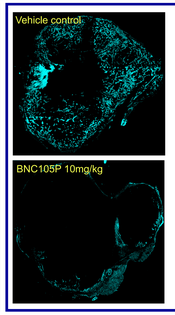

Figure 1C. Representative perfusion images of FaDu xenografts treated with vehicle control (Top) or BNC105P (Bottom)

We evaluated the activity of BNC105 in experimental models of human Head and Neck cancer. Our evaluations investigated the potency of BNC105 against the hypopharyngeal carcinoma cell line, FaDu, in *in vitro* proliferation assays. FaDu cells were exposed to BNC105 at varying concentrations for 72 hours and cell proliferation was measured by a tetrazolium-based colorimetric assay (MTS). Proliferation relative to vehicle was calculated and EC50 determined using a sigmoidal dose response curve (Figure 1A). BNC105 was found to be very active at inhibiting *in vitro* growth of FaDu cells with an EC50 of 0.29 nM.
Furthermore, the efficacy of BNC105 in disrupting the vasculature of FaDu tumors grown in Balb/c nu/nu mice was evaluated. Tumor vascular perfusion was assessed by labeling with the fluorescent dye H33342. Twenty four hours following treatment with BNC105P or vehicle control, tumor bearing animals were injected with H33342 to stain functional blood vessels. Quantitative measurement of tumor vascular perfusion was performed using Image J based analysis of tumor sections and expressed as the percentage of total tumor area labeled with H33342 (Figure 1B). Representative images of tumor vessel perfusion for BNC105P and vehicle control treated animals are shown in Figure 1C.

BNC105 shows anti-proliferative activity *in vitro* and vascular disrupting activity *in vivo* on the hypopharyngeal tumor cell line FaDu.

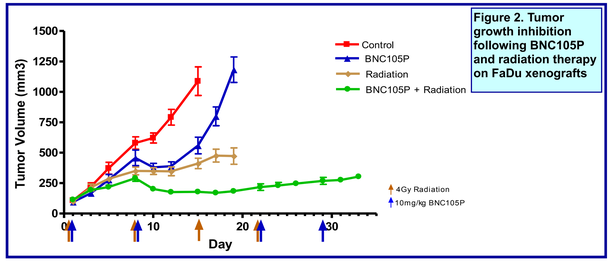

Figure 2. Tumor growth inhibition following BNC105P and radiation therapy on FaDu xenografts

To further investigate the potential therapeutic utility of BNC105 in Head and Neck cancer, evaluation was undertaken in combination with radiation, a standard therapy for Head and Neck cancer patients.

Balb/c nu/nu mice were inoculated with 2 x 10^6 FaDu cells in the hind flank. Once mean tumor size had reached $100mm^3$, mice were treated with 4 weekly doses of radiation at 4Gy, Day 1 / Day 8 doses every 21 days of BNC105P at 10mg/kg, or a combination of both treatments (n=10/group). Tumor volume measurements were taken throughout the treatment period. Tumor growth was significantly decreased when BNC105P was combined with radiation compared to either treatment alone (Figure 2). Furthermore, animals treated with radiation alone displayed a greater degree of tumor ulceration (necessitating early euthanasia on day 19) compared to animals treated with radiation in combination with BNC105P.

Combination of BNC105P treatment with radiotherapy yields improved anti-tumor efficacy in animal models of Head and Neck cancer and appears to ameliorate radiation induced ulceration.

Identification of Anti-Cancer Agents that can be Combined with BNC105 to Yield Improved Efficacy

CELL LINE	Indication	Carboplatin ED_{90}	Cisplatin ED_{90}	Doxorubicin ED_{90}	5-FU ED_{90}	Gemcitabine ED_{90}	Paclitaxel ED_{90}
MDA-MB-231	Breast adenocarcinoma						
U87-MG	Brain Glioblastoma						
HT29	Colorectal adenocarcinoma						
Calu-6	Lung carcinoma						
SKOV-3	Ovary adenocarcinoma						
BxPC-3	Pancreas adenocarcinoma						
DU145	Prostate carcinoma						
786-O	Renal cell adenocarcinoma						

CELL LINE	Indication	Oxaliplatin ED_{90}	Sorafenib ED_{90}	Tarceva ED_{90}	Sunitinib ED_{90}	Topotecan ED_{90}
MDA-MB-231	Breast adenocarcinoma					
U87-MG	Brain Glioblastoma					
Lovo	Colorectal adenocarcinoma					
Calu6	Lung carcinoma					
SKOV-3	Ovary adenocarcinoma					
FaDu	Pharynx squamous cell carcinoma					
DU145	Prostate carcinoma					
786-O	Renal cell adenocarcinoma					

Synergistic
Additive
Antagonistic
Not determined

Table 1. ED90 outcomes from combination testing of BNC105 with other chemotherapeutic agents

Human cancer cell lines were used to evaluate suitable agents for combination with BNC105. It was reasoned that synergistic or additive activity in inhibiting cancer cell proliferation *in vitro* may be indicative of a potential therapeutic benefit to be gained by combining these agents *in vivo*.

Our analyses were based on measurements of *in vitro* cell proliferation in the presence of the compounds under evaluation. Proliferation measurements were carried out by a tetrazolium-based colorimetric assay (MTS). Cells were treated with a combination of BNC105 and the compound under evaluation, or with each of these agents alone. ED90 data derived from these evaluations were analyzed using the quantitative software CaluSyn to determine the combination index (CI) (Chou 2006). Based on their CI, compounds were classified as antagonistic, additive or synergistic combination partners for BNC105 (Table 1).

BNC105 displays additive to synergistic effects with platinum based agents (cisplatin, carboplatin), 5 FU and Tarceva in several cancer cell lines. This result justifies further evaluation of these combinations *in vivo*.

In vivo Anti-Tumor Activity of BNC105 in Combination with Cisplatin

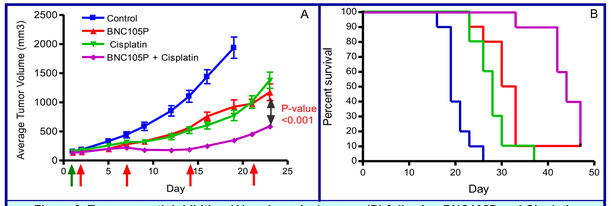

Figure 3. Tumor growth inhibition (A) and survival curves (B) following BNC105P and Cisplatin treatment on Calu-6 lung cancer xenografts in Balb/c nu/nu mice

Evaluation of combining BNC105 with cisplatin *in vitro* indicated that these two agents yield synergistic anti-proliferative effects on a number of human cancer cell lines including breast, colon, pancreatic and lung (Table 1). In order to obtain further evidence of the possible therapeutic utility of combining BNC105 with cisplatin, we conducted an *in vivo* study in mice bearing solid tumors generated with the human lung carcinoma cell line, Calu-6.
Balb/c nu/nu mice were inoculated with 2 x 10^6 Calu-6 cells subcutaneously in the axilla region. Once mean tumor size had reached $150mm^3$ mice were treated with cisplatin on day 1 at 4mg/kg, weekly with BNC105P at 10mg/kg starting on day 2, or a combination of both drugs (n=10/group). Tumor volume measurements were taken throughout the treatment period. Statistical analyses were performed on complete groups. Animal survival was assessed throughout the experiment (Figure 3B).

The synergistic effects seen with BNC105 in combination with cisplatin *in vitro* translate to improved therapeutic efficacy and survival in animal models of human lung cancer.

BNC105 is Not Susceptible to Efflux by the Polyglycoprotein (Pgp) Transporter – a Common Mechanism of Cancer Resistance to Chemotherapy

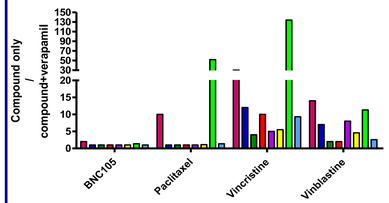

Figure 4. Effect of blocking Pgp efflux pumps with verapamil on the anti-proliferative activity of BNC105 and other tubulin targeting agents

Changes in the anti-proliferative action of BNC105 against human cancer cell lines in the presence of the Pgp transporter inhibitor, verapamil, was used as a measure of BNC105 drug efflux by Pgp. It was reasoned that if BNC105 is a substrate for Pgp, then its activity against cancer cell proliferation will be higher in the presence of verapamil.

In vitro anti-proliferative activity was measured by a tetrazolium-based colorimetric assay (MTS). Cells were seeded 24 hours prior to addition of BNC105 or other tubulin targeting drugs with and without the Pgp blocker verapamil. Following a 72 hour incubation period, MTS was added and the plate read at 492nm following a further 2 hour incubation period. The proportion of surviving cells in the presence or absence of verapamil was determined relative to control and the EC50 calculated. The effect of verapamil on drug activity was calculated as the ratio determined by dividing EC50 (compound only) by EC50 (compound+verapamil). The activity of BNC105 was not affected by verapamil-inhibition of Pgp (Figure 4). In contrast, the tubulin targeting agent vincristine displayed increased activity in the presence of verapamil. These observations demonstrate that BNC105 is not a substrate for Pgp-mediated efflux.

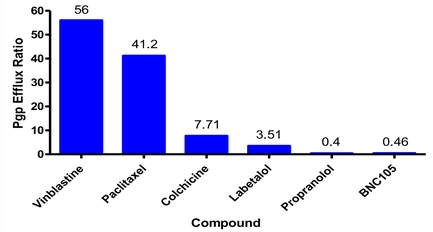

Figure 5. Efflux of BNC105 and reference compounds through MDR-MDCKII monolayers

We also investigated the potential susceptibility of BNC105 to drug transporters using cell monolayers *in vitro*. Madin-Darby canine kidney cells expressing the MDR1 (Pgp) gene (MDR1-MDCKII) were used to assess BNC105 permeability through a monolayer. BNC105 and reference compounds were placed on either the apical (A) or basal (B) side of the cell monolayer and allowed to diffuse through to the basal or apical side respectively. Permeability from A-B and B-A was calculated and a permeability ratio (BA/AB) determined.

If a compound is a Pgp substrate its movement from the basal to the apical side of the monolayer will be high, thus generating a BA/AB ratio greater than 5. If a substrate is not a Pgp substrate, it will move through the monolayer by diffusion and generate a BA/AB ratio less than 5 (Horio et al, 1989). BNC105 was found to move through the cell monolayer by passive diffusion as indicated by the ratio value of 0.2<ratio<5.

Based on these studies, it was concluded that BNC105 is not a substrate for the Pgp transporter. Therefore, BNC105 activity is not susceptible to resistance mechanisms mediated by Pgp efflux.

Summary & Conclusions

- **BNC105 exhibits anti-proliferative activity *in vitro* and vascular disrupting activity *in vivo* on the Head and Neck tumor cell line FaDu.**
- **Combination of BNC105P treatment with radiotherapy yields improved anti-tumor efficacy in Head and Neck cancer and appears to ameliorate radiation induced ulceration.**
- **BNC105 displays additive/synergistic anti-proliferative activity with platinum based agents (cisplatin, carboplatin), 5 FU and Tarceva in several human cancer cell lines.**
- **BNC105P in combination with cisplatin yields improved therapeutic efficacy and survival in an animal model of human lung cancer.**
- **BNC105 is not a substrate for the Pgp transporter. Therefore, BNC105 activity is not susceptible to resistance mechanisms mediated by Pgp efflux.**

References

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