2720. Identification and *In Vivo* Validation of Unique Anti-Oncogenic Properties and Mechanisms Involving Protein Kinase Signalling and Autophagy Mediated by the Investigational New Agent PV-10

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INTRODUCTION

- PV-10 (10% Rose Bengal) is a small-molecule agent previously shown to have potent immunotherapeutic and anti-tumor activities against a number of tumors including metastatic melanoma and refractory neuroblastoma.
- PV-10 is currently undergoing clinical testing as a single-agent for refractory metastatic neuroendocrine cancer (NCT2693067) and in combination with checkpoint inhibitors for metastatic melanoma (NCT02557321) and metastatic uveal melanoma (NCT00986661).
- We have previously determined that PV-10 induces cell death at pharmacologically relevant concentrations in a panel of phenotypically diverse adult solid tumor cell lines.[1]
  - However, the molecular concequences of this phenomenon have not yet been fully elucidated.
- The purpose of this study was to investigate the target validation and modulation of PV-10 on protein kinase signalling and their associated impact on specific oncogenic pathways in these tumor cells.

METHODS

- A panel of human tumor cell lines derived from breast (MCF-7, T-47D, MDA-MB-231), colorectal (LoVo, T-84), head and neck (CAL-27, Detroit-562, FaDu, UM-SCC-1), and testicular (NCC-IT, NTERA-2, TCAM-2) tissues were treated with PV-10 (Provectus Biopharmaceuticals Inc., Knoxville, TN) and the resulting cytotoxic effects were examined by Alamar Blue assay.
- Protein kinase profiling was performed using the human phospho-kinase antibody array (#ARY003C, R&D Systems) according to the manufacturer's protocols.
- Western blotting was used to investigate autophagic markers and protein kinase activity.
- Cell migration inhibition was determined by the wound healing assay following treatment with a sublethal dose of PV-10 or pan-WNK inhibitor WNK463.
- Tumor xenograft studies were carried out according to established protocols.

RESULTS

PV-10 delivers cytotoxic activity *in vitro* against a panel of high-risk and refractory adult solid tumor cell lines

- PV-10 is cytotoxic at pharmacologically relevant concentrations across the indicated cell lines (**Fig. 1A**).
  - Specifically, tumor cell lines originating from testicular tisisues were highly sensitive to PV-10 treatment (mean ± standard deviation IC_{50}: 37.5±16.4 µM; *n*=3) compared to breast (117.5±71.0 µM; *n*=3), colorectal (64.8±10.2 µM; *n*=3), and head and neck tissues (106.6±29.2 µM; *n*=4) (**Fig. 1B**).

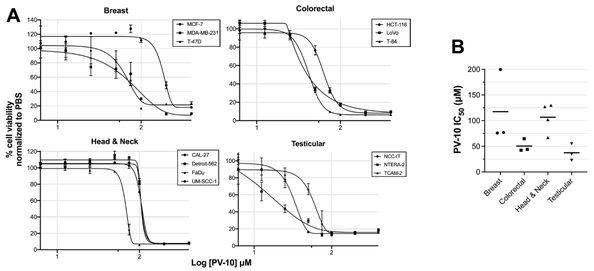

Figure 1. (A) Dose-response curves of different adult solid tumor cell lines were treated with increasing concentrations (6.25-400 µM) of PV-10 for 96 hours. Cell viability was measured by Alamar Blue assay. Percent cell viability was normalized to corresponding treatment with PBS (vehicle control). Mean percentages of cell viability were calculated from two technical replicates and standard deviations are shown. **(B) Mean distribution of PV-10 IC_{50} values (µM) for cell lines from Figure 1A.**

PV-10 treatment shows target modulation of key markers associated with apoptosis, autophagy induction

- Western blot analyses show time-dependent target modulation of (**Fig. 2**):
  - Pro-apoptotic protein markers in PARP cleavage and caspase-3 activation, indicating drug-induced apoptosis in the tested adult solid tumor cell lines.
  - Autophagic regulators, whereby the treatment of PV-10 leads to the downregulation of p62/SQSTM1, upregulation of beclin-1, and conversion of LC3B-I into LC3B-II.

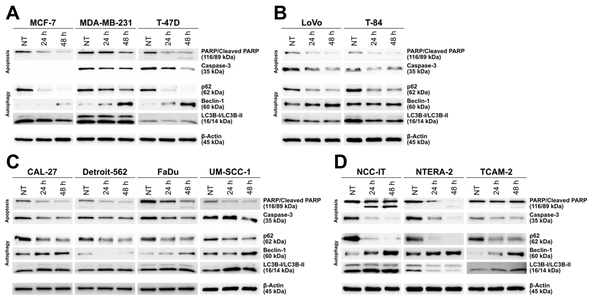

Figure 2. Western blotting of adult solid tumor cell line lysates treated with either PBS (vehicle control) or 100 µM of PV-10 for 24 and 48 hours from (A) breast, (B) colorectal, (C) head and neck, and (D) testicular tissues. Total cell lysates were prepared and analyzed by immunoblotting to detect the levels of markers associated with apoptosis (total and cleaved PARP and caspase-3) and autophagy induction (p62/SQSTM1, beclin-1, and LC3B-I/LC3B-II). β-actin was used as a loading control. Molecular masses are indicated in kilodaltons (kDa). MCF-7 cells lack the expression of caspase-3.

PV-10 targets multiple protein kinase signalling pathways, including the phosphorylation of WNK lysine deficient kinase 1 (WNK1)

- PV-10 treatment leads to consistent inhibition of WNK1 phosphorylation at the site T60 based on protein kinase profiling of drug-treated cancer cells when compared to vehicle-treated cells (**Fig. 3**).
- WNK1 has been recently recognized as an inhibitor of autophagy[2] and a promoter of cancer cell proliferation, migration, and invasion in several cancers[3-4] by potentially regulating important oncogenic pathways, including Wnt signalling (via controlling β-catenin levels).[5]

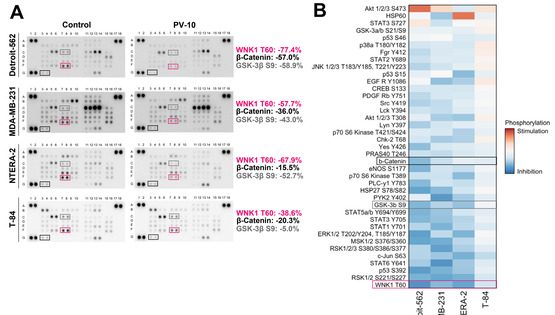

Figure 3. (A) Phosphokinase antibody array blots of whole-cell extracts. Total protein lysates from Detroit-562 (head and neck), MDA-MB-231 (breast), NTERA-2 (testicular), and T-84 (colorectal) cells treated with either PBS (vehicle control) or 100 µM of PV-10 for 3 hours were applied to a proteome phospho-kinase array. The corresponding blots for each cell line were equivalently incubated with protein (200-600 µg) and exposed for 30-60 seconds. **(B) Heat map plot of relative signal intensity (to vehicle-treated cells) following analysis of protein kinase signalling modulation by PV-10.**

PV-10 treatment inhibits the migration of cancer cells similar to the pan-WNK inhibitor WNK463

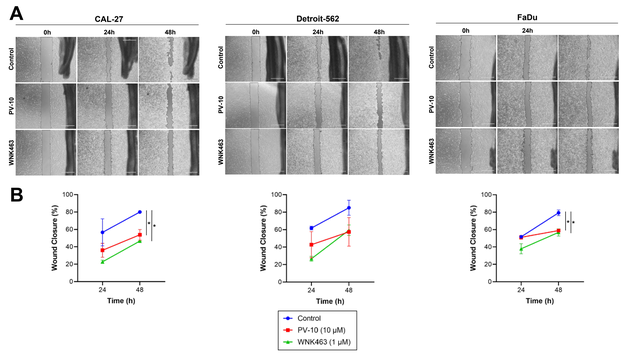

Figure 4. (A) Wound closure of adult solid tumor cell lines CAL-27, Detroit-562, and FaDu following treatment with either PBS (vehicle control), or a sublethal dose of PV-10 (10 µM) or pan-WNK inhibitor WNK463 (1 µM). Representative images were taken 24- and 48-hours post-treatment. **(B) Dot plots show quantification of relative wound area.** Mean relative wound closure (*n*=2 per treatment) and standard deviations are shown. Asterisks show significant differences, unpaired Student's *t*-test, *P*<0.05.

PV-10 treatment induces significant regression of FaDu mice xenograft tumors *in vivo*

- FaDu mice xenograft tumors (human adult hypopharyngeal carcinoma; head and neck cancer cell line) responded to treatment with PV-10 in a dose-dependent manner (**Fig. 5**).
  - For control tumors, tumor size increased from 46.2 mm^2 7 days post-treatment to 137.4 mm^2 22 days post-treatment. By comparison, size of tumors treated with 25 µL of PV-10 increased from 57.7 mm^2 to 98.6 mm^2 and size of tumors treated with 50 µL of PV-10 decreased from 64.9 mm^2 to 60.5 mm^2 (**Fig. 5A**).

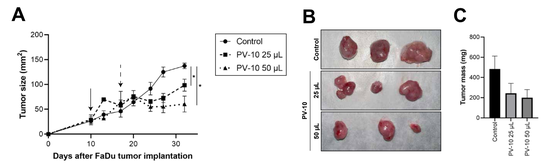

Figure 5. PV-10 induces tumor regression *in vivo*. CB17 SCID mice (*n*=3 per group) were subcutaneously injected on the right flank with FaDu cells. When tumor size was at least 25 mm^2, tumors were injected intratumorally with either 50 µL of PBS (vehicle control) or 25 or 50 µL of PV-10. **(A) FaDu tumor growth was measured using a Vernier caliper.** Solid arrow indicates the first treatment day (day 10) and dashed arrow indicates the second treatment day (day 17). Mean tumor size and standard errors of the mean are shown. Asterisks show significant differences, unpaired Student's *t*-test, *P*<0.05. **(B) Photograph of excised tumors at the experimental endpoint (day 32). (C) Tumor mass weighed following excision.** Mean tumor mass and standard errors of the mean are shown.

CONCLUSIONS

- In addition to the known activity of PV-10 to mediate tumor-specific immune responses and cytotoxic effects in neoplasms, we have identified novel therapeutic targets for PV-10, such as WNK1, and provide new insights into its effect on autophagy and metastasis.
- Our data provide essential mechanism-based evidence and biomarkers of activity for the inclusion of an effective PV-10 backbone in cancer treatment protocols.
- Additional studies are in progress to establish optimal conditions for systemic administration and effective drug combinations for the development of early phase clinical studies for the treatment of high-risk and refractory adult solid tumors.

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