Oncolytic activity of Coxsackievirus A21 (CAVATAK™) in human pancreatic cancer

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VIRALYTICS Targeted Cancer Treatment

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Abstract

Coxsackievirus A21 (CVA21: CAVATAK™) exhibits oncolytic activity both *in vitro* and *in vivo* in a number of human cancers including melanoma, multiple myeloma, breast cancer, prostate cancer and malignant glioma. CAVATAK™ is currently under clinical evaluation in patients with late stage melanoma,[1] breast cancer,[2] prostate cancer[3] and head/neck cancer. Coxsackievirus A21 requires interaction with surface expressed human ICAM-1 (intercellular adhesion molecule-1) for cellular attachment and lytic cancer cell infection. Historical immunohistochemical studies have reported high levels of ICAM-1 expressed in biopsies obtained from both localised and metastatic pancreatic cancer tumors.[4,5,6] The presented study evaluates the oncolytic potential of the genetically unmodified wild-type human CVA21, in both *in vitro* cultures and *in vivo* mouse models of human pancreatic cancer. *In vitro* studies established that ICAM-1 was expressed at relatively high levels on the surface of human pancreatic cancer cells, thereby facilitating rapid CVA21-induced lytic infection in such cancer cell cultures. *In vivo* CVA21 challenge studies employing human pancreatic cancer xenograft mouse models are discussed. Based on findings to date, administration of wild-type CVA21 may provide new therapeutic avenues for the treatment of metastatic human pancreatic cancer.

Methods

Cells:

As-PC-1, CAPAN-2 and PANC-1 cells were obtained from the American Type Culture Collection (ATCC).

As-PC-1 cells were maintained in RPMI, CAPAN-2 in McCoy's 5A Modified Media Formulation and PANC-1 cells in DMEM (all with 5-10% FCS) incubated at 37ºC with 5% CO_2. PANC-1-luc cells were generated using a lentiviral luciferase vector as described previously [7] to allow the assessment of tumor burden using bioluminescent imaging.

Virus: Coxsackievirus A21 Kuykendall strain (CAVATAK™) [Viralytics Ltd]

Tissue arrays and immunohistochemical analysis of ICAM-1 levels:

A panel of formalin-fixed normal human pancreatic tissues and cancer specimens were examined for ICAM-1 expression. Immunohistochemistry was carried out using the Vectastain peroxidase Mouse IgG ABC Kit (Vector laboratories) according to the manufacturer's protocol. The primary monoclonal antibody ICAM-1 G5 (Santa Cruz) was used at 1:1250.

Flow cytometric evaluation of ICAM-1 and DAF receptor levels:

Quantitative flow cytometry for ICAM-1 and DAF (decay accelerating factor) receptor levels was performed using QuantiBRITE™PE beads (BD #340495) as a calibrator to determine the number of antibodies bound per cell. PANC-1 cells were harvested before staining with PE-conjugated antibodies against ICAM-1 (ab18222) and DAF (ab25540)[Abcam, Sapphire Bioscience].

Evaluation of virus induced cytopathic effect (CPE):

The panel of pancreatic cancer cell lines were incubated with serially diluted CAVATAK™ inocula for 7 days and cell viability was examined by a sodium 3´-[1-(phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro)(XTT) cell proliferation assay.

One step viral growth curve of CAVATAK™ in PANC-1 and PANC-1-luc cells:

To investigate the replication of CAVATAK™ in pancreatic cancer cells, viral growth curves were established in PANC-1 and PANC-1-luc cell lines. Confluent cell monolayers in 24-well plates were infected with a multiplicity of infection (MOI) of 10 $TCID_{50}$/cell of CAVATAK™. The infection was allowed to proceed for 1 h at 37ºC before being washed 3 times with PBS. The monolayers were replenished with 1 ml of 2% FCS DMEM, and the infection was harvested at time intervals of 0, 4, 7, 16, 24, 48 and 72, 96 and 144 h. Virus supernatants were then collected and subjected to three consecutive freeze-thaw cycles, before $TCID_{50}$ assays were performed using SK-Mel-28 melanoma cells to determine the virus yield at each time point.

PANC-1-luc xenograft mouse model of oncolytic virotherapy:

Female SCID (severe combined immunodeficient) mice (n=32), were subcutaneously implanted with PANC-1-luc cells (2×10^6 cells in a volume of 100 µL). After six days of tumor establishment, mice were treated with a single intratumoral (i.t) or intravenous (i.v) injection of either saline (n=8 per group) or the oncolytic virotherapy agent, CAVATAK™ (1×10^8 $TCID_{50}$ in100 µL) (n=8 group). Eight days post tumor inoculation, mice were given a second treatment of saline or CAVATAK™ (1×10^8 $TCID_{50}$ in volumes of 100 µL). Tumor development was monitored on a weekly basis using electronic callipers. Several mice were examined using Xenogen IVIS100 bioluminescent live imaging system.

Results

1. Immunohistochemical staining of relative ICAM-1 receptor levels in biopsy samples from pancreatic cancer patients compared to normal pancreatic tissues

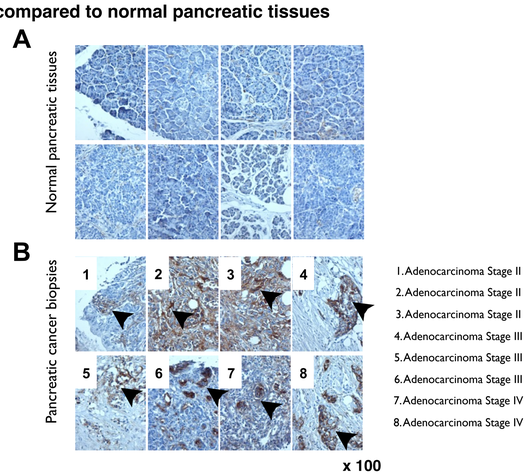

Figure 1A and B. Immunohistochemical staining (IHC) of ICAM-1 on normal pancreatic tissues (A) and pancreatic cancer biopsies (B). ICAM-1 expression was examined in a range of normal pancreatic tissues and pancreatic cancer biopsies. Representative data from each of the pancreatic cancer types are shown. The control stained tissue is shown in the top panel for comparison. Pancreatic cancer specimens stained strongly for ICAM-1 as shown by the dark brown staining (arrow), whereas in normal pancreatic tissues, weaker or no ICAM-1 staining was apparent.

2. ICAM-1 surface expression on a panel of pancreatic cancer cells

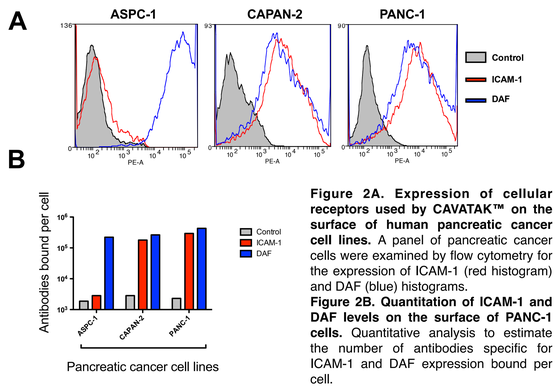

Figure 2A. Expression of cellular receptors used by CAVATAK™ on the surface of human pancreatic cancer cell lines. A panel of pancreatic cancer cells were examined by flow cytometry for the expression of ICAM-1 (red histogram) and DAF (blue) histograms.

Figure 2B. Quantitation of ICAM-1 and DAF levels on the surface of PANC-1 cells. Quantitative analysis to estimate the number of antibodies specific for ICAM-1 and DAF expression bound per cell.

3. Oncolytic activity of CAVATAK™ on a panel of human pancreatic cancer cells

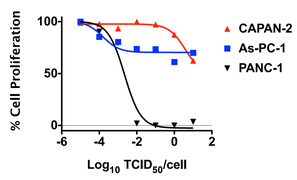

Figure 3. Dose response curves of CAVATAK™ on a panel of pancreatic cancer cell lines. PANC-1 cells appeared to be the most sensitive cell line following CAVATAK™ treatment, with no viable cells at concentrations greater than $10^{-2.31}$ $TCID_{50}$/cell. As-PC-1 and CAPAN-2 were not as sensitive as the PANC-1 cells however reduced cell proliferation to 60-70%.

4. Validation of luciferase tagged PANC-1 cell line for use *in vivo*

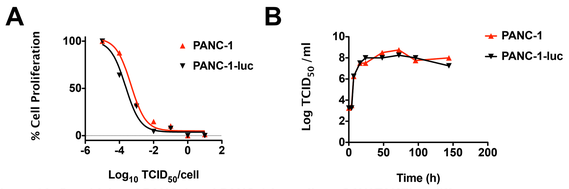

Figure 4A. Sensitivity of PANC-1 and PANC-1-luc cells to CAVATAK™ at different multiplicities of infection. PANC-1 and PANC-1-luc cell lines were infected with increasing concentrations of CAVATAK™ and were incubated at 37ºC for 72 h. Cell viability was then measured using a XTT assay. Both cell lines responded similarly to CAVATAK™ oncolysis.

Figure 4B. Viral growth curves of CAVATAK™ in PANC-1 and PANC-1-luc cells. Virus yields of CAVATAK™ were examined in both PANC-1 and PANC-1-luc cells to confirm that the introduction of luciferase failed to alter viral replication.

5. CAVATAK™ oncolytic virotherapy of PANC-1-luc tumours in SCID Balb/C mice

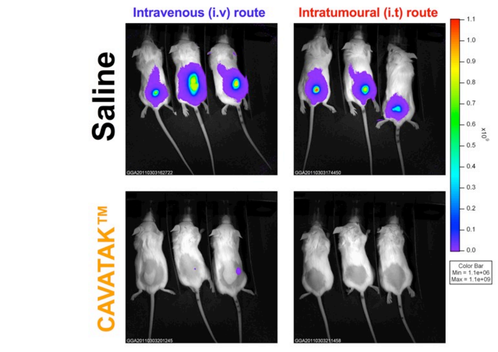

Figure 5A. Bioluminescent intensity (BLI) imaging of PANC-1 luciferase expressing tumor cells (PANC-1-luc) in a SCID Balb/C mouse model of pancreatic cancer. Mice were imaged 35 days post tumor inoculation to determine the tumor response following treatment with either saline or CAVATAK™. In both i.t and i.v. CAVATAK™ treated mice total tumor clearance was observed. In contrast, all saline treated mice displayed high levels of tumour mediated bioluminescence.

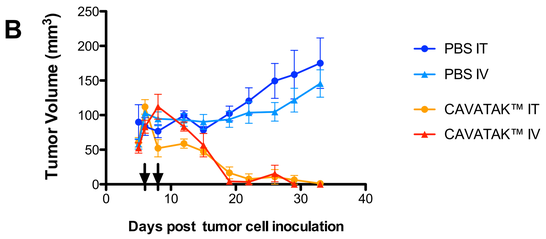

Figure 5B. Tumor volumes (mm^3) following treatment with either saline or CAVATAK™ injections (i.t or i.v). Mice were treated with two doses of intratumoral (i.t) or intravenous (i.v) injection of either saline (n=8 per group) or the oncolytic virotherapy agent, CAVATAK™ (1×10^8 $TCID_{50}$ in 100 µL) (n=8 per group). At 19 days post tumor inoculation, there was a significant difference in tumor volumes between i.t CAVATAK™ injections vs i.t saline injections ($p<0.001$) using two-way analysis of variance (ANOVA). Moreover at the same time point, the difference between i.v CAVATAK™ vs i.v saline treated mice was also statistically different ($p<0.001$) using two-way ANOVA.

Conclusions

- Immunohistochemical staining failed to detect ICAM-1 expression in biopsies of normal pancreatic tissue, while significant levels of ICAM-1 were detected in numerous pancreatic cancer biopsies from varying stages of disease.
- Moderate to high levels of cell surface ICAM-1 expression were observed following flow cytometric analysis of PANC-1 cell and CAPAN-2 cultures *in vitro*. Low levels of surface ICAM-1 were detected on As-PC-1 cells. DAF expression levels were similar across the three cell lines tested.
- PANC-1 were highly sensitive to rapid multi-cycle replication and cell cytolysis following *in vitro* challenge with CAVATAK™. CAPAN-2 were moderately sensitive, and As-PC-1 were less sensitive to CAVATAK™ challenge.
- No significant differences between the PANC-1 and PANC-1-luc cell lines in terms of sensitivity to CAVATAK™ or virus yield following initial infection were observed.
- Intratumoral or intravenous administration of two doses of CAVATAK™ to tumor bearing SCID Balb/C mice resulted in rapid cancer cell destruction and subsequent reductions in tumor burden.
- Overall these pre-clinical findings support the continued development of CAVATAK™ for the treatment of human pancreatic cancers.

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