COXSACKIEVIRUS A21 AS AN ONCOLYTIC VIROTHERAPY AGENT AGAINST MALIGNANT GLIOMA

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VIRALYTICS Targeted Cancer Treatment

Abstract

Malignant gliomas are the most common tumours of the central nervous system and respond poorly to surgery, radiotherapy and chemotherapy.[1] This disease is fatal within 1 to 2 years of onset of symptoms, despite conventional therapy. Coxsackievirus A21 (CVA21) is a naturally occurring common-cold virus that has shown potent anti-cancer (oncolytic) activity against malignant melanoma,[2] multiple myeloma,[3] as well as prostate [4] and breast cancer.[5] The selective targeting of CVA21 is based on the over-expression of the viral-cell entry receptors, intercellular adhesion molecule-1 (ICAM-1) and /or decay accelerating factor (DAF) by many cancer cells compared to normal cells. In malignant glioma, the over-expression of ICAM-1 is thought to be associated with angiogenesis and local invasiveness properties.[6] We investigated the potential use of CVA21 for the treatment of malignant glioma by confirming mRNA gene expression profiles of ICAM-1 and DAF in cancerous vs normal biopsy material, and also the presence of ICAM-1 and DAF protein expression on the surface of glioma cells using flow cytometry and immunohistochemistry. *In vitro* screening of CVA21 susceptibility was performed on a range of established and primary glioma cell lines. To further investigate the role of CVA21 in the treatment of malignant glioma, an orthotopic model of glioma was established in athymic nude mice.

Methods

Cells:

U87, U87 EGFRvIII, U373, U373 EGFRvIII glioma and normal human astrocytes (NHA) cell lines were obtained from Prof A. Guha, from the University of Toronto, Canada. The NHAs were immortalised using the human telomerase gene (hTERT). All cells were maintained in DMEM with 10% FCS at 37ºC with 5% CO_2.

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

Patient tissues:

Thirty brain tumour and four normal brain biopsy samples were obtained from the Canadian Virtual Brain Tumour Bank (CVBT) [Prof A. Guha]. The tumour samples were flash-frozen immediately after surgical excision and stored at -80ºC until further processing.

Evaluation of ICAM-1 and DAF receptor levels:

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

Relative quantitation of gene expression by qRT-PCR

Tumour and normal cellular ICAM-1, DAF and GAPDH mRNA levels (n=34) were measured using one-step qRT-PCR. Briefly, extracted RNA samples (10 ng) was used as template with the Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Mount Waverley, VIC, Australia) together with the TaqMan Gene expression assays (Applied Biosystems) for ICAM-1 (Hs00277001_m1), DAF (Hs00167090_m1) and GAPDH (Hs99999905_m1).

Immunohistochemical analysis of ICAM-1 levels:

A panel of formalin-fixed human glioma 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.

Evaluation of virus induced cytopathic effect (CPE):

Each cell line was incubated with serially diluted CAVATAK™ inocula for 72 hr and cell viability was examined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cell proliferation assay.

Orthotopic animal model of human malignant glioma:

U87 cells transfected with the luciferase gene (U87-lenti cells), were used for *in vivo* studies allowing bioluminescent monitoring of tumour burden. Female athymic nude mice (n=12), were surgically implanted with U87-lenti cells (2.2×10^5 cells in a volume of 3 µL) intracranially. After six days of tumour establishment, mice were treated with a single intracranial (i.c.) injection of either saline (n=4) or the oncolytic virotherapy agent, CAVATAK™ ($5x10^6$ $TCID_{50}$ in a volume of 5 µL) (n=8). In order to see whether a maximum tolerable dose could be achieved, n=4 mice were given multiple intraperitoneal (i.p) injections of CAVATAK™ ($5x10^6$ $TCID_{50}$/0.5 mL) per day for eight days. Tumour development was monitored on a weekly basis using the Xenogen IVIS100 bioluminescent live imaging system.

Results

1. ICAM-1 and DAF surface expression on human glioma cells

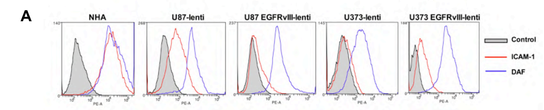

Figure 1A. Expression of cellular receptors used by CAVATAK™ on the surface of human glioma and normal human astrocyte (NHA) cell lines. A panel of human glioma and NHA cell lines were examined by flow cytometry for the expression of ICAM-1 (red histogram) and DAF (blue histogram).

Figure 1B. Quantitation of ICAM-1 and DAF levels on the surface of human glioma and NHA cell lines. Quantitative analysis to estimate the number of antibodies specific for ICAM-1 and DAF expression bound per glioma and NHA cell.

2. The study of ICAM-1 and DAF expression levels at mRNA and protein levels in biopsy samples from human glioma patients

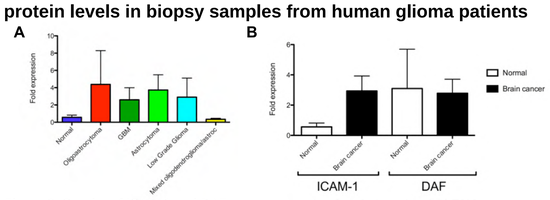

Figure 2. Graphs of ICAM-1 and DAF gene expression levels in extracted RNA samples from human glioma and normal brain tissue samples (n=34). Figure 2A shows the average expression levels of ICAM-1 mRNA relative to GAPDH in normal vs malignant biopsy material. Figure 2B shows the summary of n=4 normal tissues and n=30 brain cancer tissues for ICAM-1 and DAF gene expression relative to GAPDH.

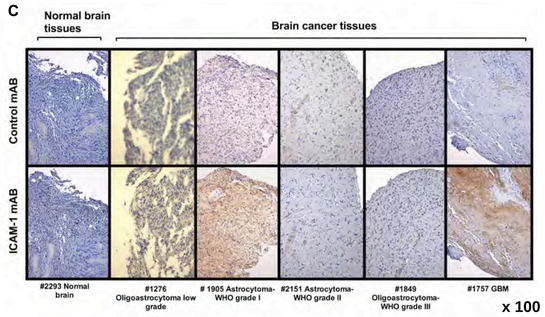

Figure 2C. Immunohistochemical staining (IHC) of ICAM-1 on normal and human glioma specimens. ICAM-1 expression was examined in a range of normal and malignant glioma formalin fixed specimens. Representative data from each of the brain cancer types is shown. The control stained tissue is shown in the top panel for comparison. GBM specimens stained strongly for ICAM-1 as shown by the dark brown staining, whereas in the normal brain tissue, astrocytomas (Grade I) and oligoastrocytomas (Grade II), weaker ICAM-1 staining was apparent.

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

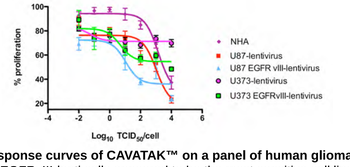

Figure 3. Dose response curves of CAVATAK™ on a panel of human glioma cell lines. U87-lenti and U87 EGFRvIII-lenti cells appeared to be the most sensitive cell lines following CAVATAK™ treatment, with approximately 23.3% viability and 24.3 % viability respectively. This is consistent with the high expression of ICAM-1 on the surface of these cells. Normal human astrocytes were shown to be sensitive to CAVATAK™ infection, however this was consistent with the expression levels of ICAM-1 found on the surface of NHAs.

4. Orthotopic glioma model in athymic nude mice

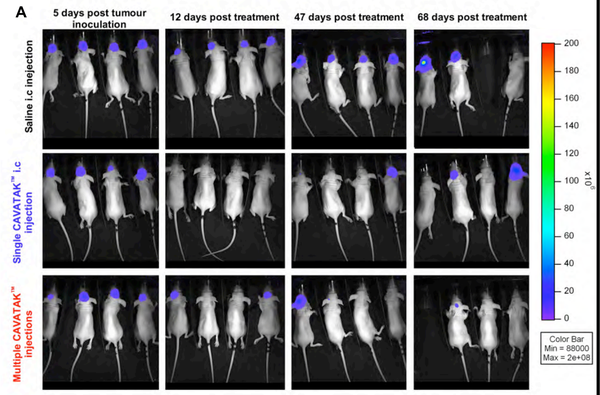

Figure 4A. Bioluminescent imaging of U87 luciferase expressing tumours (U87-lenti cells) in an athymic nude mouse model of human malignant glioma. Mice were imaged at the indicated time points to determine the tumour response following treatment with either saline or CAVATAK™. Twelve days after a single CAVATAK™ i.c injection treatment, it can be seen that 2/4 mice had total tumour clearance in the multiple CAVATAK™ injections group, 4/4 mice in the single CAVATAK™ i.c injection group, whereas saline treated U87-lenti mice showed a reasonably high bioluminescent intensity. Mice were imaged in the same sequence from left to right at each of the indicated time points.

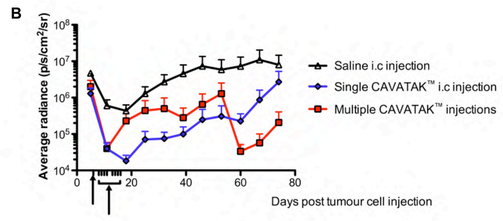

Figure 4B. Tumour response following treatment with either saline or CAVATAK™ injection(s) as measured by Xenogen imaging. There was no significant difference in tumour burden between single vs multiple injections of CAVATAK™ at any time point two-way analysis of variance (ANOVA . At day 67 post tumour inoculation, the difference between CAVATAK™ vs saline treated mice was statistically different ($p<0.05$) using two-way ANOVA.

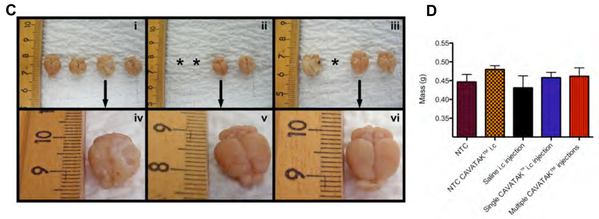

Figure 4C. Photographs of mouse brains following fixation with formalin. (i) Brains from the saline i.c treated mice, (ii) single CAVATAK™ i.c treated mice, (iii) brains from the multiple CAVATAK™ injection group (*indicates brains that were preserved in RNA later™ for further processing). (iv) Magnified view of the indicated brain from panel (i), (v) Magnified view of the indicated brain from single CAVATAK™ i.c injection group. (vi) Magnified view of the indicated brain from the multiple CAVATAK™ injection group. Destruction of the left hemisphere due to tumour growth can be seen in the saline treated mouse brain in panel iv) . **Figure 4D. Average brain weights of mice at conclusion of study.** Saline i.c injection treated mouse brain weights were slightly lower than those of the CAVATAK™ treated group however this was not statistically significant (one-way ANOVA). The injection of CAVATAK™ intracranially did not affect mouse brain weights significantly (one-way ANOVA).

Conclusions

- Quantitative flow cytometry revealed that ICAM-1 and DAF were present on both normal human astrocytes (NHA) and brain cancer cell lines. Whether the NHAs are truly representative of normal brain tissue is still questionable as these cells have been in long term culture and are immortalised with the catalytic component of telomerase (hTERT).
- A range of *ex vivo* biopsy samples from malignant glioma patients and several normal brain tissue samples were examined for ICAM-1 and DAF gene expression using quantitative RT-PCR. It was demonstrated that brain cancers had higher levels of ICAM-1 (3-fold) compared to normal brain tissues (0.6-fold) relative to GAPDH.
- Tissue obtained from localized forms of brain cancer showed increased surface ICAM-1 expression with minimal expression of ICAM-1 in normal brain tissues at the protein level as shown by immunohistochemical staining.
- From the *in vitro* analysis of CAVATAK™ infection in glioma cells, CAVATAK™ is able to infect the glioma cell lines and corresponds with the expression of the viral cell-entry receptor ICAM-1 on the cell surface.
- The *in vivo* study revealed that a single intracranial injection of $5x10^6$ $TCID_{50}$ of CAVATAK™ into athymic nude mice bearing malignant glioma tumours was effective at early time points. However regrowth was observed in some virus treated mice. Three out of four animals treated with a single i.c. injection of CAVATAK™ followed by multiple i.p injections of CAVATAK™ were tumour free upon bioluminescent imaging.
- The results from this project forms the basis for new and exciting studies into the enhancement of CAVATAK™ therapy for the treatment of malignant glioma and demonstrates a proof-of-concept for CAVATAK™ virotherapy in malignant glioma patients.

Acknowledgements

We would like to thank Research Technologist Zia Karim for his assistance with providing the tumour cell lines and histological specimens.
This project was co-funded by Cure Cancer Australia Foundation and Cancer Australia.

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