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Title: “Use of phosphoinositide analogues to enhance chemosensitivity: A novel adjuvant chemotherapeutic for medulloblastoma.”

Introduction

The project was designed to test the efficacy of novel inhibitors of AKT signaling as an adjuvant therapy for medulloblastoma in a pre-clinical setting. The following (2) specific aims were proposed:

Specific Aim 1. To characterize AKT isotype activity and signaling in medulloblastoma, and to characterize the selectivity of inhibition of AKT by phosphoinositide analogues in vitro in cellular systems modeling medulloblastomas.

Specific Aim 2. To characterize the role of AKT in resistance to chemotherapeutic agents of model medulloblastomas, and to determine the pharmacologic efficacy of AKT inhibition in enhancing the effects of standard chemotherapy.

To accomplish these aims, the first phase of the work involved completing the characterization of isotype expression and activity in medulloblastoma clinical samples and model cell lines to be used in the study. These studies were completed at the protein level by western blot analysis of tumor tissue and cell lysates. Using total RNA extracts from the same clinical tissue samples extractions were accomplished by pulverizing the 50-70 mg snap frozen tissue from our brain tumor tissue bank (VCU IRB#3031) in nuclease-free mortars and pestles and extracting protein and RNA from separate portions of powdered tissue on a bed of dry ice. This protocol has the added benefit of allowing multiple protein extraction protocols to be performed from the same sample in a scaled down format for use in AKT activity assays. We have optimized the in vitro activity assays for use in this format and are currently finalizing this portion of the work. Quantitative real-time polymerase chain reactions (qPCR, Taqman Assays) were performed for detection of AKT isotypes compared with normal brain and trauma cerebellum, the results have been prepared for publication in the first manuscript to arise from this work . This manuscript is currently in the final phases of preparation and will be submitted in 2005.

Results:

Specific Aim 1. AKT isotype expression was studied by quantifying the three known AKT/protein kinase B isotypes, AKT1, AKT2, and AKT3 at the protein and RNA levels. In a small sample set (n=13 tumors), over-expression of the AKT2 isotype was found to correlate significantly with decreased overall survival (Pearson's correlation, $r = -0.63$). In this study, protein levels also correlated with AKT activity levels detectable by western blot. The results from the AKT isotype/activity characterization study have been presented at the 2005 AACR and AANS annual meetings. Ongoing studies building on this data include

1.1. Effects of active AKT on cell signaling. Stable transfection of VC312 and D283 cells with pTet-Off and our proprietary EGFP vector has been accomplished and preliminary experiments have shown that transient and stable transfection of cDNAs in the pTRE2 vector yield robust trans-gene expression. We encountered some initial difficulty with cloning the full length AKT2 cDNA from Medulloblastoma sources, but to date have obtained the full coding sequence of AKT2 and are currently subcloning this into pTRE2. After verifying expression level and using FACS to select high EGFP expressing subclones, we will continue this work and use these cells *in vivo*. Because the set-back with cloning experiments, we employed AKT2 siRNA oligonucleotides to continue to test the relevance and role of AKT2 in PNET. AKT2 siRNA was effective in down-regulating AKT2 by western blotting and after 48 hours treatment and led to greater chemosensitization of PNET cells to both BCNU and Cisplatin. This data supports a role for AKT2 in chemo-resistance in PNET.

1.2. Effect of AKT inhibitors on purified AKT activity in vitro. The dose-response relationship of medulloblastoma cell lines with the AKT inhibitors has been accomplished *in vitro* in cellular system using AKT1. We have obtained recombinant AKT2 and AKT3 proteins from a commercial source and repeated the AKT inhibition studies. Little effect was seen on any of the purified full-length recombinant AKTs in this format. The mechanism of action of the SH-5 and SH-6 PI analogue inhibitors of AKT is thought to rely on cellular interaction with the pleckstrin homology (PH) domain, preventing phosphatidylinositol-mediated membrane recruitment of AKT isoforms and subsequent activation by membrane-associated kinases. Therefore, it is not surprising that we see little effect of these inhibitors on purified enzyme activity with recombinant proteins. We have therefore concentrated on assessing the effect of inhibitors on AKT in a cellular format using treatment of VC312 and D283 cells and immunoprecipitation kinase assays. These studies indicate that AKT1,2, and 3 isoforms all demonstrate significant down-regulation of phosphotransfer activity to GSK3 β peptide, a target of signaling by AKT isoforms. In addition, a concurrent loss in AKT enzyme phosphorylation levels was detected, assessed by comparing total protein levels for each isoform with detectable regulatory domain phosphorylation site differences before and after treatment with SH-6 in immunoprecipitated kinases. These studies indicate an effective but non-selective effect of this inhibitor class.

Specific Aim 2:

2.1. Effects of AKT inhibitors as single agents and in combination with chemotherapy agents.

The phosphoinositide analogue inhibitor SH-6 was used in combination with selected concentrations of chemotherapeutic agents BCNU and Cisplatin to assess effects on viability of the model medulloblastoma cells. The MTS and clonogenic assays were proposed for these studies. We have substituted a luminescence based ATP cell viability assay (Cell-Titer Glo CellViability Assay, Promega) in place of the MTS assay, because we have found it to be a more sensitive assay for studies quantifying viable cell number when compared with MTS cell viability assays. Drug sensitivity studies were first performed on individual chemotherapeutic drugs in a range from 0.1 to 50 μ M (BCNU),

and 0.1 to 5 μ M (Cisplatin), to assess the response of each cell line to the chemotherapeutic drugs. Similar studies were performed using SH-6 in a range from 0-30 μ M. Sensitivities of both cell lines to each drug were determined. Initial studies with SH-6 in combination with BCNU and Cisplatin, by clonogenic and ATP assays, indicate a sensitivity to the drug with doses of 10-15 μ M, administered in a single dose schedule. However, the enhancement of cell kill appears to be neither synergistic nor additive. In subsequent studies, Vincristine and Etoposide showed only minor effects on cell viability at the doses examined, and no greater than additive effects were seen in either cell line tested when co-administered with SH-6. In our hands, Cisplatin had the greatest effect on cell viability, with or without the AKT inhibitor SH-6. Work with Temozolamide is not yet complete, due to a delay in obtaining the drug in a form usable for these studies, which should be available within the next month. TMZ may show a greater synergism with SH-6 since it is one of the few chemotherapeutic drugs reported to induce significant Caspase-3 activity in brain tumors on its own. As with each drug mentioned above TMZ will be examined first to determine the LD50 dose for each cell line and then tested with an SH-6 dose regimen, and assessed by TUNEL staining (Live-Dead Apoptosis kit, Roche) and Caspase-3 activity assays (Caspase-Glo, Promega). The latter assay will be performed with Caspase inhibitor peptides to assess the involvement of individual levels of the Caspase activity cascades in the cell death mechanism. Caspase-9 and Caspase-3 inhibitors used in conjunction with Cisplatin and SH-6 were shown previously to reverse the enhancement of cell death caused by AKT inhibitor/chemotherapy drug co-treatment.

2.2. Effects of selective AKT inhibition in nude mouse model of medulloblastoma. Growth of model pediatric brain tumors in vivo.

The testing of AKT selective inhibitors as chemosensitizing agents *in vivo* is currently underway. We will continue this project by extensively characterizing our novel PNET xenotransplant (VC312R) and the D283 tumor models *in vivo*. Studies underway and supported by this grant include histopathological comparison of implanted intracranial tumors with from D283 and VC312 with snap-frozen tissues of PNET/medulloblastoma (including the patient tissue from which the VC312 cells were derived), to further characterize the AKT signaling pathway targets specifically of AKT2. We have also performed preliminary microarray analyses of surgically obtained PNET/medulloblastoma samples with either AKT2 over-expression or without over-expression, to determine potential mediators of tumor malignancy and response to therapy.

We believe this pairing of a manipulable *in vitro/in vivo* model with detailed molecular study of the tumor from which it derives (VC312), *and* an understanding of its relation to PNET on a larger scale through the microarray studies, will give us greater confidence in assessing the present and future AKT inhibitors as chemotherapeutic agents for PNET/medulloblastoma. The same suite of techniques will be applied in evaluating the next tumor explant models we are able to establish. We have applied for further funding from the National Institutes of Health and other private foundations to support this important work. The additional studies we have performed will be invaluable in assessing the molecular and cellular changes induced by the tested chemotherapy regimens and AKT inhibitors. As we continue the testing of AKT inhibitors *in vitro* and *in vivo*, we are also

assessing AKT-isotype selective inhibitors in our models, along with RNAi strategies directed specifically at down-regulating AKT2.

Manuscript preparation and final report to NBTF

Manuscript 1. (In preparation)

Van Meter TE, Tye GW, Dill D, Fillmore HL, Broaddus WC.

Elevated AKT signaling in Medulloblastomas mediated By AKT2 over-expression suppresses apoptosis. (submission Fall, 2005)

Manuscript 2. (In preparation)

Van Meter TE, Tye GW, Cash D, Broaddus CH, Broaddus WC.

AKT2 signaling suppresses chemotherapeutic response in primitive neuro-ectodermal tumors of childhood. (submission Spring, 2005)

Summary:

The specific aims of the grant were designed to assess the involvement of the AKT signaling pathway in PNET/medulloblastoma by performing detailed analysis of AKT isotype expression levels, by examining the selectivity of phosphatidylinositol drugs toward AKT1, 2 and 3, and by assessing the effect of novel AKT inhibitors on PNET response to chemotherapy *in vitro* and *in vivo*. During the grant period we were able to accomplish the characterization of AKT isotypes and signaling. Leading to the discovery that AKT2 isotype over-expression is of significance in PNET *in situ* and *in vitro*. The studies conducted on selectivity of the inhibitors indicated a non-selectivity toward specific isotypes of AKT, perhaps due to the conservation of structure within the protein domains (PH-domains) with which SH-6 interacts. Nevertheless, the co-administration of AKT inhibitor and commonly used chemotherapy drugs such as BCNU and Cisplatin demonstrated a chemosensitization to the chemotherapy, which we attribute to the role of AKT signaling in tumor cell survival pathways. This is an important first step supporting the further development of this new class of drugs, or other types of AKT inhibitor, for PNET. Assuming low toxicity of the AKT inhibitors in humans, a decrease in the effective chemotherapy dose could lead to more effective therapy and fewer debilitating side effects for patients. This funding has allowed us to develop and refine this scientific project and provided the necessary preliminary data for continued funding and pursuit of this work. AKT-selective inhibitor drugs may be of benefit for PNET and Medulloblastoma patients in the near future, which we will continue to test with an eye toward greater selectivity among the different isotypes. To date no AKT2-selective inhibitors have been described, the development of which may be of greatest benefit to PNET patients, based on the work reported here.