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A Pre-Clinical Assessment of the Pan-ERBB Inhibitor Dacomitinib in Pediatric and Adult Brain Tumors

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Abstract

Glioblastoma in adults, and medulloblastoma and pineoblastoma that mainly affect children, are aggressive brain tumors. The survival for patients with glioblastoma remains dismal. While the cure rate for medulloblastoma exceeds 70%, this figure has stagnated over the past few decades and survivors still contend with significant long-term debilitating side effects. The prognosis for pineoblastoma is age-dependent, with little chance of a cure for children younger than three years. More effective molecularly targeted strategies are urgently required to treat these cancers. Hyper-activation of epidermal growth factor receptor (EGFR) signaling is characteristic of several different classes of human cancers, including a subset of glioblastoma and medulloblastoma. This has provided the impetus for the development of a suite of EGFR pathway blockers, including second generation irreversible inhibitors, such as dacomitinib. We have developed a comprehensive drug evaluation pipeline, including *in vitro* interaction analyses and orthotopic xenograft mouse models, to address the efficacy of drugs for brain tumor treatment, enabling the exclusion of potentially ineffective treatments and prioritization of truly beneficial novel treatments for clinical trial. We used this system to examine the effects of dacomitinib as a single agent, or in combination with conventional chemotherapeutics, on the growth of human adult and pediatric brain tumor cell lines. Dacomitinib inhibited EGFR or EGFRvIII activity *in vitro* in all three tumor types tested, and as a single agent induced a modest increase in survival time for mice bearing glioblastoma, which accurately predicted human clinical trial data. For pediatric medulloblastoma, dacomitinib blocked EGFR/HER signalling in orthotopic xenografts and extended median survival as a single agent, however was antagonistic when used in combination with standard frontline medulloblastoma chemotherapies. The findings caution against the use of dacomitinib for pediatric brain tumor clinical trials.

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Introduction

Glioblastoma and medulloblastoma are the most common types of malignant brain tumor affecting adults and children, respectively. Although there has been significant progress in understanding the molecular pathogenesis of these tumor types, this has yet to translate to improved outcomes. Glioblastoma continues to have a dismal prognosis in both adults and children [1,2] and while the cure rate for medulloblastoma exceeds 70% [3], this survival rate has stagnated over the past few decades at a level well below that of other childhood cancers, such as leukemia [4]. Moreover, survivors still contend with significant

Abbreviations: 4HPC, 4-hydroperoxycyclophosphamide; CC3, cleaved caspase-3; CI, combination index; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EGFRvIII, constitutively active mutant form of the epidermal growth factor receptor; SMO, *ND2:SmoA1* transgenic mouse.

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long-term debilitating side effects. Pineoblastoma is a rare and aggressive tumor of the pineal gland, which mainly affects children. The molecular biology of this disease remains inadequately understood and the prognosis is variable depending on age; with infants having little chance of a cure, while children over the age of three years treated with radiotherapy have survival outcomes similar to medulloblastoma [5]. Standard of care frontline treatment for glioblastoma includes surgery, radiotherapy and temozolomide chemotherapy [6], while for medulloblastoma and pineoblastoma, surgery and craniospinal radiotherapy are generally combined with multiple DNA alkylators and the tubulin inhibitor, vincristine [7]. Improved outcomes for brain tumor patients depend on the development of more effective targeted therapies that not only increase survival, but also reduce treatment related side-effects, particularly for pediatric patients.

The human epidermal growth factor (EGF) family of receptor tyrosine kinases consists of four members, commonly referred to as EGFR, ERBB2, ERBB3 and ERBB4. The four proteins function as homo- or heterodimers, and interact with a variety of EGFR family ligands to regulate diverse aspects of cell growth and development in a context specific manner. Hyper-activation of EGFR signaling linked to amplification, overexpression or mutation of the EGFR family genes plays a critical role in driving the initiation and progression of several common classes or subtypes of human cancers [8,9]. For this reason, the development of new drugs and therapeutic strategies aimed at blocking EGFR signaling in cancer cells has been pursued for many years, and continues to be a major focus of research laboratories world-wide. In the context of human brain tumors, aberrant EGFR signaling has been linked to the pathogenesis of a subset of glioblastoma and medulloblastoma. Approximately 40% of glioblastomas are associated with *EGFR* amplification and overexpression, and in ~60% of these cases *EGFR* amplification is associated with deletion of exons 2-7 (referred to as the *EGFRvIII* mutation) [10]. First generation EGFR inhibitors, such as erlotinib and gefitinib, which bind reversibly to EGFR have been disappointing for the treatment of glioblastoma for various reasons including pathway redundancy, the development of resistance through downstream mutations, aberrant receptor dimerization, and difficulties crossing the blood brain barrier [11]. Overexpression of ERBB2 and/or ERBB4 occurs in a subset of medulloblastoma, although the prognostic significance of these phenomena remain controversial [12]. Earlier studies [13–17] reported poorer outcomes associated with overexpression of ERBB2 alone, or in combination with ERBB4; however, the clinical significance and efficacy of EGFR/ERBB inhibitors for the treatment of human medulloblastoma has not been comprehensively assessed.

Dacomitinib (PF299804, Pfizer) is a second-generation pan-ERBB inhibitor that irreversibly and selectively binds to the ATP binding pockets of EGFR, ERBB2 and ERBB4 at low nanomolar affinities [18]. These second-generation inhibitors are considered clinically superior to their reversible predecessors because they may block the activity of multiple receptors simultaneously and for a longer duration, while maintaining at least partial activity in the presence of mutant receptors that render first generation inhibitors ineffective [19]. Animal studies for dacomitinib have demonstrated encouraging bio-availability (>50%) and half-life (>12 hours) [20]. Recent pre-clinical data suggest that dacomitinib effectively blocked the growth of *EGFR* amplified and/or *EGFR* mutant glioblastoma cells *in vitro* and in intracranial xenografts [21,22]. Moreover, dacomitinib has shown promise for the treatment of non-small cell lung cancer, squamous cell carcinoma of the head and neck, and in a subset of glioblastomas [23–26]. The effects of dacomitinib on medulloblastoma or pineoblastoma cell growth have not been assessed.

As part of our efforts to identify more effective drugs for the treatment of brain tumors, we developed a comprehensive drug evaluation pipeline, in which the efficacy of new drugs for the treatment of brain tumors can be determined, enabling the exclusion of potentially ineffective treatments and prioritization of truly beneficial novel treatments for clinical trial. Using this system, we examined the effects of dacomitinib as a single agent, or in combination with conventional chemotherapeutics currently used in the clinic, on the growth of human adult and pediatric brain tumor cells *in vitro* and *in vivo.*

Materials and Methods

Cell Lines and Culture Conditions

The human glioblastoma cell line, U87MG, and the human medulloblastoma cell line Daoy, were purchased from the American Type Culture Collection. U87MG cells were transduced with retrovirus to express green fluorescent protein (GFP) and a puromycin acetyltransferase/luciferase fusion protein (pacLuc2) using the retroviral expression constructs MSCV-ires-GFP and MSCV-ires-pacLuc2 (U87.Luc2). U87MG cells were also transduced to express pacLuc2, GFP, and a constitutively active form of the EGF receptor (EGFRvIII) using the retroviral expression constructs MSCV-ires-pacLuc2 and MSCV-EGFRvIII-ires-GFP (U87vIII.Luc2). Retroviral constructs were kindly provided by Drs Suzanne Baker and Richard Williams of St Jude Children's Research Hospital (Memphis, TN, USA). Daoy cells were also retrovirally-transduced to express luciferase using MSCV-ires-pacLuc2 (Daoy.Luc2). Medulloblastoma and glioblastoma cells were cultured in DMEM (Gibco) supplemented with Glutamax (Invitrogen), and 10% fetal bovine serum (FBS). The human patient-derived medulloblastoma and pineoblastoma cell lines, PER547, PER452 and PER453 were gifted from Prof Ursula Kees [27], and retrovirally-transduced to express luciferase using MSCV-ires-pacLuc2 (547.Luc2, 452.Luc2 and 453.Luc2). These cells were cultured in RPMI (Gibco) supplemented with Glutamax, 1 mM sodium pyruvate (Invitrogen), non-essential amino acids (Invitrogen), 50 μM 2-mercaptoethanol (Sigma-Aldrich) and 10% FBS. Short-term GBM6 cells [28] were not modified by retrovirus and were cultured in KnockOut DMEM/F12 supplemented with Glutamax and StemPro Neural Supplement (all Gibco). Growth factors (recombinant human EGF and basic FGF, Shenandoah Biotechnology) were added at 20 ng/mL. Cells were cultured at 37°C in 5% CO_2 for all experiments.

In Vitro Drug Sensitivity Assays

Dacomitinib (Pfizer or Eurasian Chemicals) and the activated form of cyclophosphamide, 4-hydroperoxycyclophosphamide (4HPC; Toronto Research Chemicals) were dissolved in DMSO (Sigma-Aldrich). Vincristine sulfate was supplied in saline (Hospira). Cells (5,000/well) were plated in 384 well plates using a Microlab NIMBUS (Hamilton). Drug dilutions were prepared in DMSO (or saline in the case of vincristine) and further diluted in media prior to addition to cells in a combination array matrix. Cells were treated for 72 hours and incubated with alamar blue (0.6 mM resazurin, 1 mM potassium hexacyanoferrate (II) trihydrate, 1 mM potassium hexacyanoferrate (III), 2.5% methylene blue (all from Sigma-Aldrich)) for the final 6 hours of treatment. Fluorescence was detected using a Biotek Synergy Mx with excitation/emission wavelengths of 570 nm and 590 nm, respectively. Raw fluorescence data were normalized to the fluorescence measured in the DMSO control, and expressed as a percentage of control. The ED50 (effective dose resulting in 50% survival) and the factor of cells affected (Fa) were interpolated from a best-fit dose-response curve determined