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Up-regulation of cholesterol associated genes as novel resistance mechanism in glioblastoma cells in response to archazolid B

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ABSTRACT

Treatment of glioblastoma multiforme (GBM), the most common and aggressive lethal brain tumor, represents a great challenge. Despite decades of research, the survival prognosis of GBM patients is unfavorable and more effective therapeutics are sorely required. Archazolid B, a potent vacuolar H^+-ATPase inhibitor influencing cellular pH values, is a promising new compound exerting cytotoxicity in the nanomolar range on wild-type U87MG glioblastoma cells and U87MG.ΔEGFR cells transfected with a mutant epidermal growth factor receptor (EGFR) gene. Gene expression profiling using microarray technology showed that archazolid B caused drastic disturbances in cholesterol homeostasis. Cholesterol, a main component of cellular membranes, is known to be essential for GBM growth and cells bearing EGFRvIII mutation are highly dependent on exogenous cholesterol. Archazolid B caused excessive accumulation of free cholesterol within intracellular compartments thus depleting cellular cholesterol and leading to up-regulation of SREBP targeted genes, including *LDLR* and *HMGCR*, the key enzyme of cholesterol biosynthesis. This cholesterol response is considered to be a novel resistance mechanism induced by archazolid B. We surmise that re-elevation of cholesterol levels in archazolid B treated cells may be mediated by newly synthesized cholesterol, since the drug leads to endosomal/lysosomal malfunction and cholesterol accumulation.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive lethal human brain tumor. GBM accounts for >54% of all gliomas and is newly diagnosed in 3 per 100,000 people per year in the USA (Ostrom et al., 2013). Based on histological features, gliomas are categorized by the World Health Organization (WHO) in four grades (I–IV) that can be correlated with prognosis and survival (Louis et al., 2007). GBM is assigned to WHO grade IV, being the most malignant form. GBMs can occur de novo (primary form) or arise from gliomas of lower WHO grade (secondary form). Compared to secondary GBMs, the primary form typically correlates with advanced age (mean 62 years) and develops more frequently in men than in women (male:female ratio = 1.6/1) (Ohgaki et al., 2004; Ostrom et al., 2013). Standard therapy of GBM includes resection, chemotherapy and radiotherapy. Treatment of GBM is quite challenging due to the infiltration of the brain and the presence of the blood brain barrier being an obstacle for many chemotherapeutics. Despite decades of research, only carmustin (BCNU, Gliadel®) and temozolomide (TMZ) made it to standard of care (Adamson et al., 2009). Taken together, the prognosis of GBM is quite bad and most patients newly diagnosed with GBM do not survive the first year (Louis et al., 2007).

The degree of malignancy of GBMs is related to specific biologic and genetic features accounting for survival prognosis. Hallmarks of primary GBMs are EGFR amplification and mutations, loss of heterozygosity (LOH) of chromosome 10q and mutations in phosphatase and tensin homolog (PTEN). In contrast, the transformation of lower-grade tumors into secondary GBMs is mainly characterized by mutations in the tumor suppressor p53 and retinoblastoma (RB) as well as overexpression of the platelet-derived growth factor receptor (PDGFR) (Furnari et al., 2007; Wen and Kesari, 2008).

One important target in cancer therapy is the epidermal growth factor receptor (EGFR). Signaling through this receptor activates the Ras-mitogen-activated protein (MAP) kinase pathway, the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) kinase pathway and the STAT signaling pathway, thus promoting proliferation, migration, cell survival and differentiation (Lurje and Lenz, 2009). EGFR mediated signaling plays a significant role in GBM. Amplification of *EGFR* has been found in 40–50% of GBMs and is often accompanied by mutations (Nishikawa et al., 1994). The most common

Abbreviations: ArchB, archazolid B; CHO, Chinese hamster ovary; C_t, cycle threshold; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered saline; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FC, fold change; GBM, glioblastoma multiforme; HF, high fluorescence; IC_{50}, half maximal inhibitory concentration; IPA, Ingenuity Pathway Analysis; LF, low fluorescence; MFI, mean fluorescence intensity; NC, no change; PFA, paraformaldehyde; V-ATPase, vacuolar H^+-ATPase; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

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variant (EGFRvIII) leads to the expression of constitutively active EGFR and is observed in 20–50% of GBMs (Schwechheimer et al., 1995; Sugawa et al., 1990; Wikstrand et al., 1998). Considering these facts, targeting EGFR in glioblastoma seems to be promising. However, detailed studies showed that the outcome of targeting EGFR in glioblastoma therapy was rather poor due to complex molecular mechanisms in EGFR/EGFRvIII signaling that lead to drug resistance of the tumor (Lo, 2010). Harboring this EGFRvIII mutation, glioblastoma cells showed a weaker response to the EGFR tyrosine kinase inhibitor gefitinib compared to cells expressing normal EGFR (Pedersen et al., 2005). However, drug resistance conferred by EGFRvIII mutation is not only limited to kinase inhibitors, but has also been reported for drugs with a completely different mode of action, such as cisplatin (CDDP) (Nagane et al., 1998).

Archazolid B, a highly cytotoxic vacuolar H^+-ATPase (V-ATPase) inhibitor, is a macrolide originally produced by the myxobacterium *Archangium gephyra* (Sasse et al., 2003). V-ATPases are ATP-dependent proton pumps that can be found in the plasma membrane as well as in the membrane of intracellular compartments, where they play a fundamental role in pH regulation. V-ATPases acidify endosomes and lysosomes, which is necessary for endocytosis, receptor recycling and activation or degradation of proteins (Nishi and Forgac, 2002). In studies with EGFR receptor, archazolid impaired recycling processes (von Schwarzenberg et al., 2014). Recently, archazolid has been shown to overcome trastuzumab resistance in breast cancer cells by disturbing recycling of the HER2 receptor (von Schwarzenberg et al., 2014). Due to these findings, archazolid B seems to be a promising drug for targeting growth factor-driven tumors, such as GBMs.

Since drug resistance is the number one cause of cancer therapy failure, it is a fundamental necessity to evaluate defense strategies in the development of new therapeutics. The aim of this study was to find out, whether glioblastoma cells display resistance toward the V-ATPase inhibitor archazolid B. Therefore, we performed mRNA microarrays with the established human glioblastoma cell line U87MG, expressing low levels of endogenous EGFR, and the mutant cell line U87MG.ΔEGFR that harbors EGFRvIII mutation. Gene expression profiling of both cell lines was performed after 24 h and 48 h treatment with archazolid B. Our data indicated drastic disturbances in cholesterol homeostasis which were validated with further experiments and identified as a survival strategy. Furthermore, general differences between both cell lines, which might be relevant for the effectiveness of archazolid B, were analyzed by comparing the microarray datasets of U87MG and U87MG.ΔEGFR control samples.

Materials and methods

Drug. Archazolid B was kindly provided by Dr. Rolf Müller (Department of Pharmaceutical Biotechnology, Saarland University, Germany), Dr. Angelika Vollmar (Department of Pharmacy, University of Munich, Germany) and Dr. Dirk Menche (Department of Organic Chemistry, University of Bonn, Germany). Aliquots dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 μM were stored at −20 °C for up to two months. In each experiment, the DMSO content did not exceed 1%.

Cell culture. The human glioblastoma cell line U87MG and the transfected U87MG.ΔEGFR were used, which expresses constitutively active EGFR due to a deletion of exons 2 through 7. Their establishment has been reported previously (Huang et al., 1997). Both cell lines were kindly provided by Dr. W. K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA, USA). Cells were maintained in DMEM (Dulbecco's Modified Eagle) with high glucose, sodium pyruvate, GlutaMAX™ and phenol red indicator (Life Technologies, Darmstadt, Germany). Cell medium was supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 1% penicillin (100 U/mL)/streptomycin (100 μg/mL) (Life Technologies). U87MG.ΔEGFR cells were maintained in the presence of 400 μg/mL G418 disulfate salt (Sigma-Aldrich, Steinheim, Germany). Cells were grown in a humidified environment at 37 °C with 5% CO_2. They were sub-cultured twice a week up to a maximum of 10 weeks.

Cytotoxicity assay. The XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay was used to determine viability of U87MG and U87MG.ΔEGFR cells upon treatment with archazolid B. In contrast to dead cells, viable cells metabolize the tetrazolium salt XTT by the mitochondrial succinate dehydrogenase system. The product is an orange formazan compound, which can be analyzed by photometry (Scudiero et al., 1988). Briefly, cells were seeded at a density of 2500 cells/96-well and treated 24 h later with varying concentrations of archazolid B in a total volume of 100 μL/well. After 72 h incubation, an aqueous solution was prepared out of XTT sodium salt (MP Biomedicals, Germany) and phenazine methosulfate (Sigma-Aldrich) and 50 μL of it was added for 2 h to each well. The absorbance was measured at 490 nm and 655 nm and the difference between E_{490} and E_{655} was calculated using an Infinite M2000 Pro™ plate reader (Tecan, Crailsheim, Germany). By comparing treated cells to untreated cells, cell viability was evaluated. Each assay was conducted three times with three replicates each. Half maximal inhibitory concentrations (IC_{50}) were calculated for each experiment from a calibration curve by linear regression using Microsoft Excel and are represented as mean values.

mRNA microarray. U87MG and U87MG.ΔEGFR cells were seeded one day prior to treatment in 25 cm^2 flasks (for 24 h treatment: 660,000 cells; for 48 h treatment: 330,000 cells). Cells were treated for 24 h and 48 h with 20 × IC_{50} concentration of archazolid B (U87MG: 16.2 nM; U87MG.ΔEGFR: 154 nM) or DMSO solvent control (0.1%). Then, total RNA was isolated using InviTrap Spin Universal RNA Mini kit (250) (Stratec Molecular, Berlin, Germany). The experiment was performed in duplicates for treated samples and for control samples by the Genomics and Proteomics Core Facility at the German Cancer Research Center (DKFZ) in Heidelberg. The quality of total RNA was confirmed by gel analysis using the total RNA Nano chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). Only samples with RNA index values greater than 8.5 were selected for expression profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Biotin-labeled cRNA samples for hybridization on Illumina Human HT-12 v4 BeadChip arrays (Illumina, Inc.) were prepared according to Illumina's recommended sample labeling procedure based on the modified Eberwine protocol (Eberwine et al., 1992). In brief, 250–500 ng total RNA was used for complementary DNA (cDNA) synthesis, followed by an amplification/labeling step (*in vitro* transcription) to synthesize biotin-labeled cRNA according to the MessageAmp II aRNA Amplification kit (Ambion, Inc., Austin, TX). Biotin-16-UTP was purchased from Roche Applied Science, Penzberg, Germany. The cRNA was column purified according to TotalPrep RNA Amplification Kit, and eluted in 60–80 μL of water. Quality of cRNA was controlled using the RNA Nano Chip Assay on an Agilent 2100 Bioanalyzer and spectrophotometrically quantified (NanoDrop). Subsequent hybridization was performed according to the manufacturer's instruction. Microarray scanning was done using a Beadstation array scanner, setting adjusted to a scaling factor of 1 and photomultiplier tube settings at 430. Data extraction was performed for all beads individually, and outliers were removed when the median absolute deviation exceeded 2.5. Then, mean average signals and standard deviations were calculated for each probe. Data analysis was done by normalization of the signals using the quantile normalization algorithm without background subtraction, and differentially regulated genes were defined by calculating the standard deviation differences of a given probe in a one-by-one comparison of samples or groups. The data was further processed using Chipster software (The Finnish IT Center for Science CSC, Espoo, Finland). For the comparison of archazolid