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Original Article

AG311, a small molecule inhibitor of complex I and hypoxia-induced HIF-1α stabilization

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ABSTRACT

Cancer cells have a unique metabolic profile and mitochondria have been shown to play an important role in chemoresistance, tumor progression and metastases. This unique profile can be exploited by mitochondrial-targeted anticancer therapies. A small anticancer molecule, AG311, was previously shown to possess anticancer and antimetastatic activity in two cancer mouse models and to induce mitochondrial depolarization. This study defines the molecular effects of AG311 on the mitochondria to elucidate its observed efficacy. AG311 was found to competitively inhibit complex I activity at the ubiquinone-binding site. Complex I as a target for AG311 was further established by measuring oxygen consumption rate in tumor tissue isolated from AG311-treated mice. Cotreatment of cells and animals with AG311 and dichloroacetate, a pyruvate dehydrogenase kinase inhibitor that increases oxidative metabolism, resulted in synergistic cell kill and reduced tumor growth. The inhibition of mitochondrial oxygen consumption by AG311 was found to reduce HIF-1α stabilization by increasing oxygen tension in hypoxic conditions. Taken together, these results suggest that AG311 at least partially mediates its antitumor effect through inhibition of complex I, which could be exploited in its use as an anticancer agent.

Introduction

In addition to genetic and epigenetic heterogeneity, the concept of metabolic heterogeneity has increased recently. Considerable heterogeneity exists between different tumors, but also within the same tumor. This intratumoral heterogeneity is predominately mediated by genetic diversity, energy demand, and the proximity to vasculature, dictating glucose and oxygen availability. Until recently, cancer cells were thought to rely only on glycolysis for ATP production, even in the presence of sufficient oxygen supply [1], but metabolic heterogeneity demonstrates that there are multiple metabolic phenotypes, going beyond the upregulated glycolysis as observed by Warburg. One of these metabolic phenotypes is mitochondrial respiration, as cancer cells have been shown to have functional mitochondria capable of producing ATP [2–4].

Anticancer agents acting through mitochondrial inhibition have been described. For example, the anti-diabetic biguanides metformin and phenformin have been shown to exert their anticancer effect by inhibiting complex I, the first enzyme in the mitochondrial electron transport chain (ETC) [5–8]. BAY-87-2243, another complex I inhibitor, has shown efficacy in a preclinical model of resistant melanoma [9]. The small molecule, IACS-10759, was specifically designed as a complex I inhibitor to target

Abbreviations: NADH, nicotinamide adenine dinucleotide; RTK, receptor tyrosine kinase; TS, thymidylate synthase; CCS, cosmic calf serum; DMSO, dimethyl sulfoxide; HMEC, human microvascular endothelial cells; TMRM, tetramethylrhodamine methyl ester; AMPK, AMP-activated protein kinase; DCA, dichloroacetate; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; α-KG, α-ketoglutarate; TCA, tricarboxylic acid; ETC, electron transport chain; HIF-1α, hypoxia-inducible factor-1 alpha; VEGF, vascular endothelial growth factor; CA9, carbonic anhydrase 9; TKI, tyrosine kinase inhibitor; PHD, prolyl hydroxylase domain; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptors; VEGFR, vascular endothelial growth factor receptor; OCT1, organic cation transporter 1; PD-L1, programmed death-ligand 1.

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chemoresistant dormant tumors [9,10]. Complex I, NADH-ubiquinone oxidoreductase, is a crucial player in mitochondrial respiration. It transfers electrons from NADH to reduce ubiquinone to ubiquinol resulting in proton translocation into the mitochondrial intermembrane space, which establishes an electrochemical gradient that is used for ATP synthesis. In order for this process to continue, molecular oxygen is required as the final electron acceptor. Regions of solid tumors often exhibit low oxygen tension (hypoxia), which has been shown to be involved in tumor development, chemo- and radioresistance, and metastasis in addition to tumor bioenergetics [11,12]. These alterations in bioenergetic processes are mediated in part by increasing gene expression involved in glycolysis and by lowering the activity of the electron transport chain [13–15]. The major cellular adaptive response to hypoxia is tightly regulated by hypoxia-inducible transcription factor-1α, HIF-1α, which is stabilized in low oxygen tensions, thus an inhibition of complex I can prevent electron transfer and decrease oxygen consumption, which in turn could decrease HIF-1α stabilization [16,17]. HIF-1α is capable of inducing a broad range of cellular responses including angiogenesis, resistance to apoptosis and tumor energetics/metabolism. Due to the importance of mitochondrial oxidative phosphorylation in hypoxia and in supporting tumor growth, progression and metastasis [18,19], the development of mitochondrial inhibitors seems well justified.

Herein we investigate the molecular mechanism of a small molecule antitumor agent, AG311. We have previously shown that AG311 significantly reduced primary tumor growth and lung metastases in two breast cancer mouse models, by 81–85% [20]. Upon further investigation, a distinct metabolic mechanism for AG311 emerged. We reported that AG311 rapidly induced necrotic cell death, depolarized the mitochondrial membrane and severely reduced intracellular ATP levels [20]. In the current study, we further define the molecular and antitumor mechanisms and identify complex I of the electron transport chain (ETC) as a likely molecular target of AG311 in isolated cells and in tumors. We show that as a downstream consequence of complex I inhibition, AG311 reduced hypoxia-induced HIF-1α stabilization. Additionally, we show that AG311 synergizes with dichloroacetate (DCA) to increase cell death in cancer cells and increase tumor volume in a xenograft tumor mouse model.

Materials & methods

Additional information is described in the supplemental methods

Cell culture

Cancer cell lines were purchased from American Type Culture Collection (Manassas, VA), except MDA-MB-435, which was a kind gift of Dr. Janet Price at MD Anderson Cancer Center in the mid-1990s. MDA-MB-435 are poorly differentiated basal-like breast cancer cells that express melanocytic molecular markers [21–23]. Cell lines were maintained as previously described [20]. Hypoxia culture was performed in 1.5% O_2 with 5% CO_2 in Invivo$_2$400 hypoxia chamber (Ruskinn, Leeds, UK).

Viability assay

Cells were treated with AG311 or for the synergy experiment treated in combination with dichloroacetate (DCA) (SigmaAldrich, St. Louis, MO) at various concentrations. The drugs were diluted in OptiMEM (LifeTechnologies) at the final concentration. After 24 or 48 h, viability was determined with PrestoBlue (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and read with a microplate reader (BioTek, Winooski, VT). For the nutrient deprivation experiment, glucose was replaced with 25 mM mannitol (SigmaAldrich) for osmolarity control. Combination index (CI) values were determined using Chou-Talalay method with CompuSyn software [24]. Where noted, total live cell count was determined by labeling nuclei with Hoechst 33342 (2 μg/ml) (LifeTechnologies) and dead cells with the membrane impermeable dye SytoxGreen (0.5 μM) (LifeTechnologies) followed by imaging with Operetta High-Content Imaging System (PerkinElmer, Waltham, MA)

Cytotoxic compound

The initial design and synthesis of AG311 was conducted by A. Gangjee and N. Zaware [25]. For cell culture experiments, AG311 was prepared as a 50 mM stock and diluted in cell culture medium with a final DMSO concentration not exceeding 0.2%.

Cellular ATP content measurement

Cells were treated with AG311 in OptiMEM for the indicated durations. The cells were lysed in 20 μL OptiMEM with 20 μL lysis buffer per well and 10 μL of lysate was added to assess ATP levels using ATP Detection Assay Kit (Abcam, Cambridge, MA) according to manufacturer's instructions. The data were normalized to ATP content of untreated cells.

Mitochondrial membrane potential

Mixed- or mono-culture was prepared as previously described [20]. For mixed culture, HMEC-1 and MDA-MB-435 cells were incubated with Cell Tracker Green (1 μM) or Cell Tracker Blue (25 μM) (LifeTechnologies). Cells were incubated with TMRM at 10 nM (Biotium, Hayward, CA) in OptiMEM for 30 min, followed by treatment with AG311 and imaging with Operetta.

Electron transport chain enzymatic analyses

The enzymatic activity of complex I, III, IV of the electron transport chain was measured spectrophotometrically in the presence of AG311 or solvent as previously described [26].

MDA-MB-435 orthotopic xenograft

The animal experiments described in this study were approved by the Institutional Animal Care and Use Committee of OUHSC. The detailed methods for this model have been described previously [27]. Five days after tumor implantation, nu/nu mice were treated intraperitoneally twice weekly with AG311 (37 mg/kg) or solvent control (described in supplemental methods). DCA was administered in the drinking water at 50 mg/kg.

Oxygraphic measurements using Seahorse

Cells (MDA-MB-435) were seeded in Seahorse Flux Analyzer 96-well plate (6500 cells/well) coated with Cell-Tak adhesive coating (Corning, Corning, NY) and incubated overnight at 37 °C, 5% CO_2 in DMEM. Cell media was replaced with XF Assay Medium containing 25 mM glucose. Seahorse XFe96 analyzer was used to record OCR (oxygen consumption rate) in response to injection of diluted solvent (DMSO) or AG311.

Oxygraphic measurements using Oroboros

Freshly isolated tumor tissue from untreated or AG311-treated mice was prepared for oxygraphic measurements as previously described [28,29]. Tumors from AG311-treated mice were excised 1 h after intraperitoneal injection with a 45 mg/kg dose.

Western blot

Western blots were incubated with HIF-1α antibody (1:1000, #3716, Cell Signaling, Danvers, MA), p-αAMPK antibody (1:1000, #2535, Cell Signaling), αAMPK antibody (1:1000, #2603, Cell Signaling) overnight at 4 °C. Levels of β-actin (Cell Signaling #4970) (1:1000) or vinculin (SigmaAldrich) (1:4000) were used as the loading control. Densitometry was performed using Image Lab software v5.2.1 (Bio-Rad, Hercules, CA). Values are represented as the ratio of target protein to loading control. For the HIF-1α blot using MDA-MB-435 cell lysate, unrelated lanes between the AG311-treated samples and $CoCl_2$ control were removed. The image was spliced together at that position.

Semi-quantitative real-time polymerase chain reaction (PCR)

Real-time PCR was performed with cDNA as described previously [30]. Quantitative values of target genes for hypoxic conditions were first normalized to 28S rRNA content using the ddCt method [31] and then normalized to the corresponding normoxic control.

Immunocytochemistry

Immunocytochemistry was performed using isothiocyanate (FITC)-labeled anti-pimonidazole antibody (Hypoxyprobe Inc, Burlington, MA) [32].