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GDC-0941 enhances the lysosomal compartment via TFEB and primes glioblastoma cells to lysosomal membrane permeabilization and cell death

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ABSTRACT

Since phosphatidylinositol-3-kinase (PI3K) inhibitors are primarily cytostatic against glioblastoma, we searched for new drug combinations. Here, we discover that the PI3K inhibitor GDC-0941 acts in concert with the natural compound B10, a glycosylated derivative of betulinic acid, to induce cell death in glioblastoma cells. Importantly, parallel experiments in primary glioblastoma cultures similarly show that GDC-0941 and B10 cooperate to trigger cell death, underscoring the clinical relevance of this finding. Molecular studies revealed that treatment with GDC-0941 stimulates the expression and nuclear translocation of Transcription Factor EB (TFEB), a master regulator of lysosomal biogenesis, the lysosomal membrane marker LAMP-1 and the mature form of cathepsin B. Also, GDC-0941 triggers a time-dependent increase of the lysosomal compartment in a TFEB-dependent manner, since knockdown of TFEB significantly reduces this GDC-0941-stimulated lysosomal enhancement. Importantly, GDC-0941 cooperates with B10 to trigger lysosomal membrane permeabilization, leading to increased activation of Bax, loss of mitochondrial membrane potential (MMP), caspase-3 activation and cell death. Addition of the cathepsin B inhibitor CA-074me reduces Bax activation, loss of MMP, caspase-3 activation and cell death upon treatment with GDC-0941/B10. By comparison, knockdown of caspase-3 or the broad-range caspase inhibitor zVAD.fmk inhibits GDC-0941/B10-induced DNA fragmentation, but does not prevent cell death, thus pointing to both caspase-dependent and -independent pathways. By identifying the combination of GDC-0941 and B10 as a new, potent strategy to trigger cell death in glioblastoma cells, our findings have important implications for the development of novel treatment approaches for glioblastoma.

1. Introduction

Glioblastoma is the most frequent primary malignant tumor of the brain that bears still a very poor prognosis despite intensive treatment regimens [1]. This highlights the need to design new treatment approaches. One promising strategy is to target perturbations in signaling pathways that are crucial for cell survival and treatment resistance of glioblastoma. For example, alterations in the phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway frequently occur in glioblastoma [2,3] and abnormal activity of this pathway has been shown to correlate with adverse clinical outcome in glioblastoma [4].

The PI3K/Akt/mTOR pathway integrates extra- and intracellular survival signals to stimulate cell growth and to block cell death [5,6]. For example, PI3K/Akt signaling may mediate its anti-apoptotic activities by changing the ratio of pro- and anti-apoptotic proteins and by interfering with mitochondrial apoptosis [7]. The mitochondrial (intrinsic) pathway of apoptosis involves mitochondrial outer membrane permeabilization (MOMP), cytochrome c release into the cytosol and caspase activation [8]. MOMP can be initiated by e.g. activation of Bax via a conformational change [8]. In addition, PI3K/Akt signaling has been reported to regulate the activity and stability of lysosomes, although the molecular mechanisms have remained elusive [9]. The lysosome plays a central role in cellular homeostasis, e.g. by regulating cellular clearance and cell death in response to environmental cues. Recently, the Transcription Factor EB (TFEB) has been identified as a master

Abbreviations: FCS, fetal calf serum; LMP, lysosomal membrane permeabilization; MMP, mitochondrial membrane potential; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol-3-kinase; TFEB, Transcription Factor EB; zVAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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regulator that controls lysosomal biogenesis [10]. The activity of TFEB and its nuclear translocation is regulated by its phosphorylation status [11,12]. Under nutrient supply, phosphorylation of TFEB, for example via mTORC1, maintains TFEB in the cytosol and inhibits its nuclear translocation [12]. Starvation or pharmacological inhibition of mTORC1 results in dephosphorylation and activation of TFEB, allowing its translocation to the nucleus to activate a transcriptional program that boosts lysosomal function [12]. For example, lysosomal protein hydrolases such as cathepsins are among the most relevant targets of TFEB [10]. Lysosomal membrane permeabilization (LMP) results in the release of lysosomal enzymes such as cathepsins from the lysosomal lumen to the cytosol [13,14]. LMP can induce apoptotic cell death featuring caspase activation and mitochondrial outer membrane permeabilization, as lysosomal enzymes can contribute to Bax activation and caspase cleavage [13,15]. Alternatively, LMP and lysosomal enzymes can trigger non-apoptotic cell death depending on the extent of LMP and the cell type [13].

The PI3K/Akt/mTOR pathway is currently considered as a promising cancer drug target, since it is aberrantly activated in most human cancers including glioblastoma [16]. We previously reported that PI3K inhibitors as single agents exhibit little cytotoxicity against glioblastoma cells, whereas they sensitize for chemotherapy- or death receptor-induced apoptosis [17,18]. This indicates that PI3K inhibitor-based combination regimens are required for optimal therapeutic effects. Recently, we reported that the natural compound B10, a glycosylated derivative of betulinic acid, triggers cell death with apoptotic and non-apoptotic features by destabilizing lysosomes [19]. Searching for new strategies to potentiate the antitumor activity of PI3K inhibitors, we tested the PI3K inhibitor GDC-0941 in combination with B10 in the present study.

2. Materials and methods

2.1. Cell culture and chemicals

Glioblastoma cell lines were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1 mmol/L glutamine (Biochrom), 1% penicillin/streptomycin (Biochrom), and 25 mmol/L HEPES (Biochrom). To obtain primary tumor-derived cell lines, primary tumor material was first dissociated mechanically. After centrifugation, cells were mildly trypsinized using TrypLE Express (Invitrogen, Darmstadt, Germany), filtered through a 70 μM cell strainer and cultured in serum-free DMEM/Ham's F12 medium (Life Technologies, Inc.), supplemented with B27 supplement without Vitamin A (Invitrogen), 1% penicillin/streptomycin and 2% Fungizone (Invitrogen). N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was purchased from Bachem (Weil am Rhein, Germany), CA-074me from Sigma (Taufkirchen, Germany). GDC-0941 was kindly provided by Genentech Inc. (San Francisco, USA) and B10 by BioService Halle (Halle, Germany). Other chemicals were purchased from Sigma unless otherwise indicated. Cells were pretreated with GDC-0941 for 24 h, followed by addition of B10 or inhibitors for indicated time points.

2.2. Determination of apoptosis and cell viability

Apoptosis was determined by fluorescence-activated cell-sorting analysis (FACScan, BD Bioscience, Heidelberg, Germany) of DNA fragmentation of propidium iodide (PI)-stained nuclei as described previously [20]. Cell viability was determined by crystal violet staining. Briefly, adherent cells were stained for 10 min at room temperature using a crystal violet solution containing 0.5% crystal violet, 30% ethanol and 3% paraformaldehyde. The plates were then washed in water and crystal violet incorporated by the cells was re-solubilized in a solution containing 1% SDS. Absorbance at 550 nm was measured using a microplate reader (EL800, Biotek, Bad Friedrichshall, Germany). Results are expressed as percentage of cell density relative to the untreated control prior to stimulation.

2.3. Western blot analysis

Western blot analysis was carried out as described previously [20] using the following antibodies: rabbit anti-caspase-3 (Cell Signaling, Beverly, USA), mouse anti-CD107a (LAMP-1) (BD Pharmingen, Heidelberg, Germany), goat anti-TFEB and mouse anti-cathepsin B (Abcam, Cambridge, USA) and rabbit anti-BaxNT (Upstate Biotechnology, Lake Placid, USA), followed by goat anti-mouse immunoglobulin G (IgG) or goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, USA). Mouse anti-β-actin (Sigma) or mouse anti-α-tubulin (Calbiochem, Darmstadt, Germany) were used as loading controls. Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). Representative blots of at least two independent experiments are shown.

2.4. Knockdown of caspase-3, Bax and TFEB by RNA interference

Glioblastoma cells were seeded at 0.12×10^6 per 6-well tissue culture plate and allowed to settle for 24 h. Cells were transfected with caspase-3 Stealth RNAi (HSS101373, Invitrogen), Bax Stealth RNAi (HSS141355 and HSS141356, Invitrogen), TFEB Stealth RNAi (HSS111670, Invitrogen) or esRNAi (EHU059261, Sigma) or non-targeting universal negative control siRNA (Invitrogen) using TransMessenger transfection reagent (Qiagen, Hilden, Germany), in a total volume of 1150 μL/well. Transfection medium was replaced after 3 h and incubated for additional 72 h. Cells were reseeded and treated with GDC-0941 and B10 as described before.

2.5. Lysotracker staining

Lysosomal levels were determined in living, non-fixed, non-lysed cells using LysotrackerRed (50 nM, Invitrogen). Cells were incubated with LysotrackerRed for 10 min, washed once with PBS and immediately analyzed by flow cytometry.

2.6. Nuclear fractionation assay

Cells were trypsinized, washed and mechanically lysed in nuclear extraction buffer (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl, 0.5 mM DTT; 1 mM Natrium-Vanadat and Protease inhibitor). The supernatant after the first centrifugation step reflected the cytosolic fraction, the remaining pellet was washed and resuspended in the corresponding buffer and displayed the nuclear fraction.

2.7. RNA extraction and cDNA synthesis

RNA was extracted from samples either treated with GDC-9041 or after transfection with non-targeting universal negative control siRNA or TFEB Stealth RNAi by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of RNA was quantified by photometry. 1 μg of RNA was used as a template for the following cDNA synthesis using the ImProm-II™ Reverse Transcription System.

2.8. Real-time polymerase chain reaction using SYBR Green I

The real-time PCRs were set up in LightCycler Capillaries according to the manufacturer's instructions using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany) in a reaction volume of 20 μL qSTAR qPCR primer pairs of TFEB (OriGene, Rockville, USA) were used at a concentration of 5 μM and 1 μg of cDNA was used for amplification. Real-time PCR analysis was performed using a LightCycler 2.0. The thermal profile used was as followed: 40 cycles; 15 s 95 °C, 30 s 58 °C and 30 s 72 °C. The SYBR Green I fluorescent signal was determined for each cycle at the end of the extension step.

2.9. Determination of mitochondrial membrane potential

To determine mitochondrial membrane potential, cells were incubated with 100 ng/mL tetramethylrhodamine methylester perchlorate (TMRM) (Sigma) for 10 min at 37 °C, trypsinized, washed once with PBS and immediately analyzed by flow cytometry.

2.10. Immunoprecipitation of active Bax

Bax activation was determined by immunoprecipitation of Bax using an active conformation-specific antibody as previously described [21]. Briefly, cells were lysed in CHAPS lysis buffer (10 mmol/L HEPES, pH 7.4; 150 mmol/L NaCl; 1% CHAPS). An amount of 1 mg protein was incubated with 8 μg mouse anti-Bax antibody (clone 6A7, Sigma) overnight at 4 °C followed by addition of 10 μL pan-mouse IgG Dynabeads (Dako, Hamburg, Germany), incubated for 2 h at 4 °C, washed with CHAPS lysis buffer, and analyzed by Western blotting using rabbit anti-BaxNT antibody (Upstate Biotechnology).

2.11. Statistical analysis

Statistical analysis was assessed by Student's t test (2-tailed distribution, 2-sample, unequal variance).