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Original Articles

DNA damage response and anti-apoptotic proteins predict radiosensitization efficacy of HDAC inhibitors SAHA and LBH589 in patient-derived glioblastoma cells

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ABSTRACT

HDAC inhibitors have radiosensitizing effects in established cancer cell lines. This study was conducted to compare the efficacy of SAHA, LBH589, Valproic Acid (VPA), MS275 and Scriptaid in the patient-derived glioblastoma model. In more detail, SAHA and LBH589 were evaluated to determine predictors of response. Acetylated-histone-H3, γH2AX/53BP1, (p)Chek2/ATM, Bcl-2/Bcl-XL, $p21^{CIP1/WAF1}$ and caspase-3/7 were studied in relation to response. SAHA sensitized 50% of cultures, LBH589 45%, VPA and Scriptaid 40% and MS275 60%. Differences after treatment with SAHA/RTx or LBH589/RTx in a sensitive and resistant culture were increased acetylated-H3, caspase-3/7 and prolonged DNA damage repair γH2AX/53BP1 foci. pChek2 was found to be associated with both SAHA/RTx and LBH589/RTx response with a positive predictive value (PPV) of 90%. Bcl-XL had a PPV of 100% for LBH589/RTx response. Incubation with HDACi 24 and 48 hours pre-RTx resulted in the best efficacy of combination treatment. In conclusion a subset of patient-derived glioblastoma cultures were sensitive to HDACi/RTx. For SAHA and LBH589 responses were strongly associated with pChek2 and Bcl-XL, which warrant further clinical exploration. Additional information on responsiveness was obtained by DNA damage response markers and apoptosis related proteins.

Introduction

Glioblastoma is the most malignant form of primary brain tumor, originating from the supporting tissue of the brain, the glial cells. With the current standard treatment consisting of maximal safe surgical resection followed by temozolomide and radiation therapy (RTx) patients have a post-operative expected survival of 12–15 months [1,2]. Since glioblastoma is highly resistant to conventional anticancer regimens, new combined approaches are urgently needed to improve outcome. Histone deacetylase inhibitors (HDACi) are anticancer drugs that alter the epigenome by inhibiting histone deacetylases which are involved in the deacetylation of core histones. Hereby these drugs change gene expression, but they also affect non-histone proteins directly [3,4]. The HDACi SAHA, LBH589, Valproic Acid (VPA), Scriptaid and MS275 are well-studied drugs and effectively sensitize various tumor types to radiation (RTx) as was shown in several *in vitro* and *in vivo* models including conventional glioma cell lines [5–10]. SAHA and LBH589 are currently tested in clinical trials as combination drugs for temozolomide or RTx in glioblastoma [11]. The mechanism of action of HDACi as radiosensitizers is a combination of chromatin relaxation, altered transcription of DNA damage repair genes and common cell death pathway synergisms [4].

In contrast to the established glioma cell lines, patient-derived glioblastoma cultures that are cultured under serum-free conditions resemble the genotype of the parental tumor and as a consequence reflect the genetic heterogeneity between patients [12]. Therefore, this study investigated the responsiveness to various HDACi in combination with RTx in the patient-derived glioblastoma model. This representative model allows determination of tumor subtypes related to treatment response and identification of molecules related to response. In conventional cell lines, important molecules for the mechanism of action of HDACi were found to be acetylated histones, cell cycle regulatory proteins

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($p21^{CIP1/WAF1}$), Bcl-2 family apoptotic proteins and the DNA damage response [13–16]. The objective of this study was to assess sensitivity of patient-derived glioblastoma cultures to five HDACi in combination with RTx. These HDACi included SAHA, LBH589, VPA, Scriptaid and MS275. Also, we identified molecules that were associated with response to the clinically relevant HDACi SAHA and LBH589 as radiosensitizers. Furthermore, the optimal timing of HDACi/RTx was assessed, as well as the effects of fractionated radiation on combination treatment.

Materials and methods

Chemicals

The compounds SAHA and MS275 were obtained from Cayman chemicals (MI, USA), VPA from Sigma-Aldrich (MO, USA), LBH589 from Biovision (CA, USA), Scriptaid from Santa Cruz Biotechnology (CA, USA) and Staurosporin was obtained from BioMol (Germany). Stocks were prepared at 100 mM (VPA) in sterile water and at 50 mM (SAHA), 10 mM (Scriptaid), 4 mM (MS275) and 200 μM (LBH589) in dimethyl sulfoxide (Sigma-Aldrich) and stored at –20 °C. The DMSO concentration was kept below 1% in the treatment dilutions.

Patient-derived glioblastoma cell cultures

Fresh tumor tissue specimens were obtained by surgical resection at the Department of Neurosurgery of the ErasmusMC (Rotterdam, The Netherlands) and Elisabeth Hospital (Tilburg, The Netherlands). Tumor material was obtained with patients' informed consent as approved by the institutional review board of the ErasmusMC. The tumor tissue specimens were dissociated, maintained as patient-derived glioblastoma cultures under serum-free conditions, and characterized as was described previously [12]. Twenty-two patient-derived glioblastoma cultures were used for the experiments. The (clinical) characteristics of the original tumors are shown in Table 1. These clinical characteristics were investigated for relation with the mean differences in viability of single agent treatment compared to either combination treatment (SAHA/RTx or LBH589/RTx).

Viability assays

Dose–response assays for the HDACi alone were performed to determine the IC_{50} values by using at least three different drug concentrations. The combination treatment was applied using at least two different HDACi concentrations, 24 hours before applying single dose RTx (3 Gy, Cs-137 source). The concentrations of HDACi were (0.25)/0.5/1.0 μM SAHA; (0.1)/0.3/0.6 mM VPA; (0.35)/0.7/1.4 μM Scriptaid; (0.05)/0.1/0.5 μM MS275; (1)/5/20 nM LBH589. These concentrations were based on the values determined in the dose–response assays in monotherapy, and the highest dose to be tested had to be between the average IC_{20} and IC_{50} of all cultures. This was used to determine enhancement in combination with 3 Gy RTx. One dose of RTx was tested, as patients in the clinical setting receive a fixed dose of RTx daily. The dose of 3 Gy RTx was chosen based on the initial screen of the panel of cultures. The latter showed that 3Gy was the only fixed dose where RTx still had a small effect on the most resistant culture, and where the most sensitive culture had enough viability (>25%) at day eight after treatment to be able to study enhancement. The 96-well plates were coated with Matrigel (1:20, BD Biosciences, CA, USA) and seeded at 1×10^3 cells/well. Cell viability was measured on day eight by CellTiter-Glo assay [17] (Promega, WI, USA). The IC_{50} values were calculated by median effect equation [18]. To study the optimal sequence of HDACi/RTx, 0.75×10^3 cells/well were plated and treated with SAHA at 48 hours or 24 hours pre-RTx, simultaneously, or 24 hours post-RTx.

Western blot

The four patient-derived glioblastoma cultures GS79, GS160, GS186 and GS257 were treated with SAHA, LBH589, RTx or the combinations, according to the schedule used in the previous experiments. The cells were harvested 24 hours after RTx. For the experiment shown in Figs. 3C and 4A, the glioblastoma cultures were collected 30 minutes after RTx, together with corresponding untreated controls. Cells were washed with PBS and collected in a lysis buffer under protease and phosphatase inhibitory conditions. Protein concentrations were assessed by BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, MA, USA). Separation was performed on a 10% Acrylamide/Bis gel (Bio-Rad, CA, USA) and blotted onto a PVDF membrane (Immobilon-P, Millipore, MA, USA) using the Mini-Protean Tetra Cell system (Bio-Rad). Membranes were blocked in a 5% non-fatty milk solution 0.5 hour at room temperature. The blots were probed with anti-acetylated histone H3 (anti-ac-H3), anti-β-actin (1:300; and 1:5000, Millipore), anti-Bcl-2, anti-BclXL (both 1:500; Cell Signalling), anti-$p21^{CIP1/WAF1}$ (1:500, BD Bioscience), anti-pChek2-Thr68, anti-Chek2, ATM, pATM-Ser1981 (1:500, Cell Signalling) in 5% BSA/TBS-T overnight. After washing, membranes were incubated with secondary anti-rabbit-HRP or anti-mouse-HRP (1:2000, Dako Denmark A/S, Denmark) 1 hour at room temperature. The blots were visualized using the ChemiDoc MP system (Bio-Rad), and analyzed with the ImageLab Software (Bio-rad).

Immunohistochemistry

The patient-derived glioblastoma cultures GS79 (responsive to HDACi/RTx) and GS257 (resistant to HDACi/RTx) were selected to study the DNA repair response. Double strand breaks were identified by quantifying 53BP1 and γH2AX foci. Cells

Table 1
The characteristics of the twenty-two patient-derived glioblastoma cultures.

Glioblastoma culture	MGMT status	PFS (mths)	Gender	Age (yrs) at resection	KPS pre-operative	Recurrent tumor	Treated tumor
GS102	M	5	M	68.0	90	No	No
GS160	UM	1.5	M	58.1	90	No	No
GS184	M	2.5	M	50.4	90	No	No
GS186	M	3	F	48.2	90	Yes	TMZ/RTx
GS203	M	7	M	64.0	100	No	No
GS216	UM	2.5	F	42.9	90	Yes	TMZ/RTx
GS224	M	3.5	M	58.5	80	Yes	TMZ/RTx
GS245	UM	5	M	69.0	70	No	No
GS257	UM	2	F	60.1	60	No	RTx
GS261	M	2.5	M	82.2	80	No	No
GS274	M	6	F	67.1	70	No	No
GS279	M	19	M	62.8	90	Yes	TMZ/RTx
GS289	UM	3	M	79.6	80	No	No
GS295	UM	7	M	50.1	90	No	No
GS330	M	0	F	77.1	70	No	No
GS335	UM	3	F	60.6	80	Yes	TMZ/RTx
GS357	M	3	M	56.9	90	No	No
GS359	M	14	M	69.7	90	No	No
GS365	UM	3.5	M	59.5	90	No	No
GS401	M	2.5	M	52.6	70	Yes	TMZ/RTx
GS423	M	*	M	65.0	90	No	No
GS79	UM	4	F	74.7	90	No	No

The clinical characteristics of twenty-two patient-derived glioblastoma cultures were tested for the response to SAHA/RTx and LBH589/RTx, and of which 12 were used for testing the effects of VPA/RTx, Scriptaid/RTx and MS275/RTx. MGMT status = MGMT promoter methylation status; M = methylated; UM = unmethylated; PFS = progression free survival (months); gender, M = male, F = female; age (yrs) in years at time of resection; KPS = Karnofsky Performance Scale; recurrent tumor = if original tumor of which culture was derived was recurrent (yes) or not (no); treated tumor = if original tumor was treated in case of recurrence, with temozolomide (TMZ) and/or radiation (RTx).
*These data are unavailable.

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