Ca\(^{2+}\)-Activated IK K\(^{+}\) Channel Blockade Radiosensitizes Glioblastoma Cells

Benjamin Stegen\(^1\), Lena Butz\(^1,2\), Lukas Klumpp\(^1,3\), Daniel Zips\(^1\), Klaus Dittmann\(^4\), Peter Ruth\(^2\), and Stephan M. Huber\(^1\)

Abstract

Ca\(^{2+}\)-activated K\(^{+}\) channels, such as BK and IK channels, have been proposed to fulfill pivotal functions in neoplastic transformation, malignant progression, and brain infiltration of glioblastoma cells. Here, the ionizing radiation (IR) effect of IK K\(^{+}\) channel targeting was tested in human glioblastoma cells. IK channels were inhibited pharmacologically by TRAM-34 or genetically by knockdown, cells were irradiated with 6 MV photons and IK channel activity, Ca\(^{2+}\) signaling, cell cycling, residual double-strand breaks, and clonogenic survival were determined. In addition, the radiosensitizing effect of TRAM-34 was analyzed in vivo in ectopic tumors. Moreover, The Cancer Genome Atlas (TCGA) was queried to expose the dependence of IK mRNA abundance on overall survival (OS) of patients with glioma. Results indicate that radiation increased the activity of IK channels, modified Ca\(^{2+}\) signaling, and induced a G\(_2\)-M cell-cycle arrest. TRAM-34 decreased the IR-induced accumulation in G\(_2\)-M arrest and increased the number of γH2AX foci post-IR, suggesting that TRAM-34 mediated an increase of residual DNA double-strand breaks. Mechanistically, IK knockdown abolished the TRAM-34 effects indicating the IK specificity of TRAM-34. Finally, TRAM-34 radiosensitized ectopic glioblastoma in vivo and high IK mRNA abundance associated with shorter patient OS in low-grade glioma and glioblastoma.

Implications: Together, these data support a cell-cycle regulatory function for IK K\(^{+}\) channels, and combined therapy using IK channel targeting and radiation is a new strategy for anti-glioblastoma therapy. Mol Cancer Res; 13(9); 1283–95. ©2015 AACR.

Introduction

Glioblastoma multiforme (GBM) represents the most common primary brain tumor in adults. The therapeutic concept combines resection of the tumor followed by adjuvant radiation therapy combined with simultaneous temozolomide chemotherapy. Although the administration of the alkylating cytostatic agent significantly prolongs overall survival (OS), the prognosis of patients with glioblastoma remains very poor, with a median survival time of less than 2 years (1).

The underlying radiobiological mechanisms of the poor radiation response of glioblastoma appear to include multiple factors. Among those are low cellular radiation sensitivity, high proportion of cancer stem cells, enhanced repopulation, protective tumor microenvironment, infiltration of the tumor by immune cells, and highly migratory phenotype of the GBM cells giving rise to infiltrative tumor growth. In addition, glioblastoma cells have been proposed to evade therapy by persisting in potential subventricular neural stem cell niches outside of the radiation target volume (2).

Glioblastoma cells functionally express high numbers of Ca\(^{2+}\)-activated IK K\(^{+}\) channels (other names are hIKCa1, hKCa4, hSK4, KCa3.1) in their plasma membrane (3–6). Notably, IK channels are low expressed or even absent in human astrocytes (7) but upregulated during neoplastic transformation and malignant progression of the glioma (8). This suggests a specific function of these channels in glioblastoma tumorigenesis. As a matter of fact, IK channels have been demonstrated to be indispensable for glioblastoma cell migration (for review, see ref. 9). Accordingly, IK protein expression in the tumor significantly correlates with poor survival of the patients with glioma (10). Similar to glioblastoma, IK channels are upregulated in a variety of further tumor entities such as prostate (11), breast (12), and pancreatic cancer (13) as well as lymphoma (14) where they have been proven to control cell cycle and tumor growth.

In addition to tumor cell migration and proliferation, K\(^{+}\) channel activity may contribute to radioresistance of tumor cells (for review, see refs. 15–17). Remarkably, the fungicide clotrimazole has been shown to impair glioblastoma growth in vitro and in vivo (18, 19) and to promote apoptotic cell death of irradiated glioblastoma cells in vitro (20). Because clotrimazole is a potent IK channel inhibitor, we tested in the present study for a functional significance of IK channels in the radioresistance of glioblastoma cells in vitro. We could show by physiologic and cell biologic means that ionizing radiation activates IK channels in glioblastoma cells. Channel activation, in turn, contributes to the cellular stress response. Accordingly, inhibition or silencing of IK channels resulted in impaired cell-cycle arrest and DNA repair and decreased the clonogenic survival of irradiated glioblastoma cells. In addition, pharmacologic targeting of IK channels radiosensitized glioblastoma grown ectopically in mice during fractionated radiation therapy.
Furthermore, a Cancer Genome Atlas (TCGA) query suggests an association between glioma IK mRNA abundance and progression-free survival (PFS) of patients with glioma.

Materials and Methods

Cell culture

Human T98G and U87MG glioblastoma cells were from the ATCC and were grown in 10% FCS-supplemented RPMI-1640 medium as described (21). The human SVGA fetal astrocyte cell line has been kindly provided by Professor Walter J. Atwood, Brown University (Providence, RI), and was maintained in 10% FCS-supplemented DMEM. Exponential growing T98G and U87 MG cells were irradiated with 6 MV photons (IR, single dose of 0, 2, 4, and 6 Gy) by using a linear accelerator (LINAC SL25 Philips) at a dose rate of 4 Gy/min at room temperature. Following 2, 4, and 6 Gy) by using a linear accelerator (LINAC SL25 Philips) at a dose rate of 4 Gy/min at room temperature. Following irradiation, cells were postincubated in RPMI-1640/10% FCS-supplemented DMEM. Exponential growing T98G and U87

Immunofluorescence

Subconfluent T98G glioblastoma cells and SVGA fetal astrocytes grown on object slides (Millicell EZ SLIDES; Millipore) were fixed with 4% paraformaldehyde in PBS for 1 hour and washed three times for 5 minutes with PBS. Cells were blocked for 1 hour with PBS containing 0.3% Triton X-100, 5% normal goat serum, and washed for 15 minutes with PBS. Incubation with rabbit anti-IK antibody (H-120, SantaCruz Biotechnology, Inc.; sc-32949, 1:50) and rabbit IgG isotype control antibody (1:250, Millipore), respectively, in antibody dilution buffer (PBS, 0.3% Triton X-100, 1% BSA) was performed for 1 hour at room temperature. Cells were washed three times for 5 minutes in PBS and incubated for 2 hours at room temperature in the dark with FITC-conjugated goat anti-rabbit IgG antibody (1:500, Novus Biologicals). Cells were washed three times with PBS, and object slides were mounted with cover slips using the DNA-specific fluorescence DAPI-containing ECTASHEILD mounting medium with DAPI (Vectashield, Vector Laboratories, BIOZOL).

Patch-clamp recording

Semicontiguous cells were irradiated with 0 Gy (SVGA, T98G) or 2 Gy (T98G). Whole-cell and on-cell currents were evoked by 9 to 11 (whole-cell) or 41 (on-cell) voltage square pulses (700 ms each) from −50 to 0 mV holding potential to voltages between −100 and +100 mV delivered in 5 or 20 mV increments. The liquid junction potentials between the pipette and the bath solutions were estimated according to previously published data (22), and data were corrected for the estimated liquid junction potentials. Cells were superfused at 37°C temperature with NaCl solution (125 mmol/L NaCl, 32 mmol/L HEPES, 5 mmol/L KCl, 5 mmol/L D-glucose, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, titrated with NaOH to pH 7.4). In the whole-cell experiment shown in Fig. 1, ionomycin (2.5 μmol/L) and TRAM-34 (1 μmol/L) or ionomycin (2.5 μmol/L), paxiline (5 μmol/L), and TRAM-34 (1 μmol/L, all from Sigma-Aldrich) were sequentially added to the bath solution. For this recording, a K-glutonate pipette solution was used containing: 140 mmol/L K-glutonate, 5 mmol/L HEPES, 5 mmol/L MgCl₂, 1 mmol/L K₂EGTA, 1 mmol/L K₂ATP, titrated with KOH to pH 7.4. In the on-cell experiments (Fig. 2), the pipette solution contained 0 or 0.01 mmol/L TRAM-34 in DMISO, 130 mmol/L KCl, 32 mmol/L HEPES, 5 mmol/L D-glucose, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, titrated with NaOH to pH 7.4. Whole-cell and macroscopic on-cell currents were analyzed by averaging the currents between 100 and 700 ms of each square pulse.

Western blotting

Surface proteins of irradiated (0 and 2 Gy) T98G cells were enriched by the use of a cell surface protein isolation kit (Pierce) according to the supplied protocol. Whole protein lysates were prepared from stably transfected T98G cells (see below). Proteins were lysed in a buffer containing 50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 10 mmol/L NaF, 2 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), additionally containing 1% Triton X-100, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 3 μg/mL pepstatin, and separated by SDS-PAGE under reducing conditions. Segregated proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Roth). Blots were blocked in TBS buffer containing 0.05% Tween-20 and 5% non-fat dry milk for 1 hour at room temperature. The membrane was incubated overnight at 4°C with the following primary antibodies in TBS-Tween/5% milk against IK (H-120, sc-32949 SantaCruz, 1:500) or the α5 subunit of the Na+ pump (Cell Signaling #3010, New England Biolabs; 1:500). Equal gel loading was verified by an antibody against β-actin (mouse anti-β-actin antibody, clone AC-74, Sigma #A2222, 1:20,000). Antibody binding was detected with a horseradish peroxidase–linked goat anti-rabbit or horse anti-mouse IgG antibody (Cell Signaling #7074 and #7076, respectively; 1:2,000 dilution in TBS-Tween/5% milk) incubated for 1 hour at room temperature, and enhanced chemiluminescence (ECL Western blotting analysis system, GE Healthcare/Amersham-Biosciences) of indicated protein levels were quantified by densitometry using ImageJ software (ImageJ 1.40g, NIH, Bethesda, MD).

Fura-2 Ca²⁺ imaging

Fluorescence measurements were performed using an inverted phase-contrast microscope (Axiovert 100; Zeiss). Fluorescence was evoked by a filter wheel (Visiport Systems)-mediated alternative excitation at 340/36 or 373/11 nm (AHF, Analyseotechnik). Excitation and emission lights were deflected by a dichroic mirror (409/16 nm beamsplitter, AHF) into the objective (Fluar x40/1.30 oil; Zeiss) and transmitted to the camera (Visiport Systems), respectively. Emitted fluorescence intensity was recorded at 587/35 nm (AHF). Excitation was controlled and data acquired by Metalfuor computer software (Universal Imaging). The 340/380-nm fluorescence ratio was used as a measure of cytosolic free Ca²⁺ concentration ([Ca²⁺]cyt). T98G cells were irradiated (0 or 2 Gy) and loaded with fura-2/AM (2 μmol/L for 30 minutes at 37°C; Molecular Probes) in RPMI-1640/10% FCS medium. [Ca²⁺]free was determined 2 to 3 hours post-IR at 37°C during superfusion with NaCl solution (125 mmol/L NaCl, 32 mmol/L HEPES, 5 mmol/L KCl, 5 mmol/L D-glucose, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂, titrated with NaOH to pH 7.4), during extracellular Ca²⁺ removal in EGTA-buffered NaCl solution (125 mmol/L NaCl,
32 mmol/L HEPES, 5 mmol/L KCl, 5 mmol/L D-glucose, 1 mmol/L MgCl2, 0.6 mmol/L EGTA, titrated with NaOH to pH 7.4), and during Ca2+ re-addition in CaCl2-containing NaCl solution.

Flow cytometry

T98G cells were preincubated (0.25 hours), irradiated (0 or 2 Gy), and incubated for further 6 hours in RPMI-1640/10% FCS medium additionally containing the base analogue 5-ethynyl-2'-deoxyuridine (EdU; 5 μmol/L). EdU incorporation was analyzed by the use of a EdU flow cytometry kit (BCK-FC488, baseklick) after fixing the cells and co-staining the DNA with propidium iodide (PI; Sigma-Aldrich) according to the manufacturer’s instructions. EdU-specific fluorescence and PI fluorescence were measured by flow cytometry (FACS Calibur, Becton Dickinson; 488 nm excitation wavelength) in fluorescence channel FL-1 (log scale, 515–545 nm emission wavelength) and FL-3 (linear scale, >670 nm emission wavelength), respectively. In additional experiments, T98G cells were preincubated (30 minutes),
irradiated (0, 2, 4, or 6 Gy), and incubated for further 24 or 48 hours in RPMI-1640/10% FCS medium additionally containing either TRAM-34 (10 μmol/L) or vehicle alone (0.1% DMSO). For cell-cycle analysis, cells were permeabilized and stained (30 minutes at room temperature) with PI solution (containing 0.1% Na citrate, 0.1% Triton X-100, 10 μg/mL PI in PBS), and the DNA amount was analyzed by flow cytometry in fluorescence channel FL-3 (linear scale). Data were analyzed with the FCS Express 3 software (De Novo Software).

γH2AX foci formation

T98G cells cultured on CultureSlides (Becton Dickinson) in RPMI-1640/10% FCS medium were irradiated (0 or 2 Gy) post-incubated for 24 hours in the presence of TRAM-34 (10 μmol/L) or vehicle alone (0.1% DMSO) and fixed with 70% ice-cold ethanol. For immunofluorescent analysis, cells were incubated with anti-γH2AX antibody (Upstate, Millipore; clone JBW301; 1:500) at room temperature for 2 hours. Positive foci were visualized by incubation with a 1:500 dilution of Alexa488-labeled goat anti–mouse serum (Molecular Probes) for 30 minutes. CultureSlides were mounted in Vectashield/DAPI (Vector Laboratories) and evaluated by conventional fluorescence microscopy.

IK shRNA

IK was downregulated in T98G cells by stable transfection with IK-specific and—for control—nontargeting shRNA using MISSION pLKO.1 lentiviral transduction particles

Figure 2.

IR increases the activity of IK K+ channels. A, experimental setup: macroscopic on-cell currents were recorded from control and irradiated T98G cells with KCl pipette and NaCl bath solution applying the depicted pulse protocol. Currents obtained in the presence and absence of the IK channel inhibitor TRAM-34 (10 μmol/L) were compared between unpaired experiments. B, macroscopic on-cell current tracings recorded during voltage square pulses to −50 and +50 mV, respectively (as shown by the gray pulse protocol in A) from control (left) and irradiated (2 Gy) T98G cells with (lower traces) and without (upper traces) TRAM-34 in the pipette solutions. Note that the prominent single-channel current deflections are generated by BK K+ channels, which are also activated by IR as reported (ref. 21; also evident from E, right). C, dependence of the mean macroscopic on-cell currents (±SE) on holding potential in control (open circles) and 2 Gy-irradiated T98G cells (2.5–5.5 hours after irradiation, closed triangles) recorded in the absence (left, n = 26–28) and presence (right, n = 8–9) of TRAM-34 in the pipette. D, mean (±SE) radiation-induced current fractions as calculated from the data in C for control (closed triangles) and TRAM-34-treated (open circles) T98G cells. E, mean (±SE) inward (left) and outward (right) conductance as calculated from the data in C by linear regression (voltage ranges are indicated by gray lines) for control (open bars) and irradiated (closed bars) T98G cells recorded in the absence (first and second bars) or presence of TRAM-34 (third and fourth bars; * and ** indicate P < 0.01 and P < 0.05, respectively, Kruskal-Wallis nonparametric ANOVA test).
IK Targeting in Glioblastoma

Figure 3.
IR modulates the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{free}}\)) but not the surface expression of IK channels in T98G cells. A, immunoblots of surface proteins from control (0 Gy) and irradiated T98G cells (2 Gy) probed against IK (top) and for loading control against the \(\alpha_2\) subunit of the Na\(^+\) pump (bottom). B, time course of the mean (±SE) cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{free}}\)) as measured by ratiometric fura-2 Ca\(^{2+}\) imaging 3 to 5 hours after irradiation with 0 Gy (open circles; \(n = 32\)) or 2 Gy (closed triangles \(n = 24\)) during removal and re-addition of external Ca\(^{2+}\). C, mean (±SE) steady-state [Ca\(^{2+}\)]\(_{\text{free}}\) (top, as indicated by gray lines at the beginning of the records in B) and mean (±SE) decrease in [Ca\(^{2+}\)]\(_{\text{free}}\) (bottom) upon removal of extracellular Ca\(^{2+}\) (as indicated by the gray arrows in B) in control (open bars) and irradiated cells (data from B; \(* P < 0.001\), two-tailed t test).

Quantitative RT-PCR
mRNAs of stably transfected T98G cells were isolated (Qiagen RNA extraction kit) and reversely transcribed in cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche). IK \(K^+\) channel and GAPDH-specific fragments were amplified by the use of SYBR Green–based quantitative real-time PCR (QT00003780 and QT01192646 QuantitTect Primer Assay and QuantiFast SYBR Green PCR Kit, Qiagen) in a Roche LightCycler Instrument.

Colonies formation assay
To test for clonogenic survival, U87MG, parental T98G, and stably transfected T98G cells (clones #2 and #3) were irradiated (0, 2, 4, or 6 Gy) in RPMI-1640/10% FCS medium additionally containing TRAM-34 (10 \(\mu\)mol/L) or vehicle alone (0.1% DMSO). After 24 hours of incubation with the inhibitor/vehicle, cells were detached, 200 to 800 cells were reseeded in inhibitor-free medium – containing TRAM-34 (10 \(\mu\)mol/L) or vehicle, and allowed to grow for further 2 to 3 weeks. The plating efficiency was defined by dividing the number of colonies by the number of plated cells. Survival fractions as calculated by dividing the plating efficiency of the irradiated cells by those of the unirradiated controls were fitted by the use of the linear quadratic equation.

Ectopic mouse model of human glioblastoma
All experiments were performed according to the German Animal Protection Law and approved by the local authorities (RP Tübingen, reference number FZ3/13). Human U87MG cells (500,000 cells in 100 \(\mu\)L PBS) were injected subcutaneously in the upper outer right hind limb of 8-week-old female NMRINu/Nu mice. Tumor growth was monitored at least 3 times per week by measuring tumor size in 3 dimensions using calipers. Upon reaching a tumor volume of around 150 \(\mu\)L, mice were randomly assigned to 4 treatment arms (control, fractionated radiation, TRAM-34, and TRAM-34 combined with fractionated radiation).

Beginning with day 0, tumors were locally irradiated under isoflurane anesthesia at room temperature with 5 consecutive daily fractions of 0 (control) or 4 Gy 6 MV photons as described (23). Six hours before each radiation fraction, mice received intraperitoneal injections of the IK channel inhibitor TRAM-34 (0 or 120 mg/kg body weight in Myglol). The drug TRAM-34 at the applied dose and local fractionated irradiation of the ectopic glioblastoma was well tolerated by the mice.

Querying TGCA datasets
Via the cBioportal Web resource (24, 25), the provisional Glioblastoma-Multiforme and Lower-Grade-Glioma TCGA databases (http://cancergenome.nih.gov/) were queried for IK mRNA abundance of the tumor specimens and PFS of the patients with glioma. In lower grade glioma and glioblastoma, 14 to 22 of 116 bases (http://cancergenome.nih.gov/) were queried for IK mRNA abundance in T98G than in SVGA cells. To estimate the abundance in T98G than in SVGA cells. To estimate the

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functionality of IK K⁺ channels in SVGA, T98G, and a further glioblastoma cell line (U87MG), currents through the plasma membrane were recorded with the patch-clamp technique in whole-cell mode with physiologic bath and pipette solutions. Records were obtained before and after Ca²⁺ permeabilizing the plasma membrane with the Ca²⁺ ionophore ionomycin (2.5 μmol/L). To characterize the Ca²⁺-stimulated current fraction, the BK K⁺ channel inhibitor paxilline (5 μmol/L) and/or the IK K⁺ channel inhibitor TRAM-34 (1 μmol/L) were added sequentially to the ionomycin-containing bathing solution. In SVGA astrocytes, ionomycin failed to induce outwardly rectifying whole-cell currents at voltages more negative than −20 mV in 6 of 6 tested cells suggestive of the absence of functional IK channel in the plasma membrane. Accordingly, bath application of TRAM-34 did not inhibit a fraction of the whole-cell currents. A representative experiment is depicted in Fig. 1B and C.

In sharp contrast, ionomycin activated a whole-cell outward current in T98G cells at all voltages more positive than K⁺ equilibrium potential (Eₖ ≈ −90 mV; Fig. 1D and E, open triangles). Paxilline inhibited about 80% of the outward current in Ca²⁺-permeabilized T98G cells (Fig. 1D and E, gray filled triangles). Additional application of TRAM-34 blocked almost all of the remaining paxilline-insensitive current fraction (Fig. 1D and E, black filled triangles). This TRAM-34-sensitive current fraction (Fig. 1F) exhibited inward rectification with a conductance density of about 100 pS/pF at negative voltages and had a reversal potential close to Eₖ. Together, these data indicate functional expression of a Ca²⁺-activated, inwardly rectifying K⁺-selective and TRAM-34–sensitive current fraction, which is characteristic for an IK current (26) in T98G glioblastoma cells but not in the astrocyte cell line. Ca²⁺-permeabilized U87MG cells showed similarly high IK channel activity albeit having lower paxilline-sensitive currents (data not shown).

To test whether IR induces changes in IK channel activity, T98G cells were irradiated with 2-Gy 6-MV photons by the use of a linear accelerator, postincubated for 2 to 6 hours, and recorded in cell-attached mode using a KCl solution in the pipette (Fig. 2A). IR stimulated an increase in the inward and outward fraction of the macroscopic cell-attached currents (Fig. 2B, top, and C, left). Importantly, when in unpaired experiments the IK channel inhibitor TRAM-34 (10 μmol/L) was added to the pipette solution (Fig. 2B, bottom, and C, right), an IR-stimulated inward current was no more detectable (Fig. 2C and D) indicative of an IR-stimulated IK current. Reportedly, IR may modulate the Ca²⁺ signaling (for review, see ref. 15). To define signaling events upstream of IK channel
activation, cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{free}}\)]) was assessed by fura-2 Ca\(^{2+}\) imaging experiments in control and irradiated (2 Gy) T98G cells 3 to 5 h after IR. In addition, IK surface expression was analyzed in control and irradiated T98G cells by immunoblots of biotinylated and avidin-separated surface proteins probed against IK and for loading control against the \(\alpha_1\) subunit of the Na\(^{+}\) pump. As shown in Fig. 3, the IR-induced increase in IK activity in T98G was probably due to IR-induced increase in [Ca\(^{2+}\)\(_{\text{free}}\)] (Fig. 3B and C) rather than to an elevated surface expression of IK channels (Fig. 3A). IR (2 Gy) induced a significant increase in steady-state [Ca\(^{2+}\)\(_{\text{free}}\)] (Fig. 3B and C, top). Upon removal and re-addition of extracellular Ca\(^{2+}\), irradiated cells showed a larger drop-down and larger re-increase of [Ca\(^{2+}\)\(_{\text{free}}\)] as compared with unirradiated cells (Fig. 3B and C, bottom). This suggests that a shift in the Ca\(^{2+}\) leak/pump equilibrium of the plasma membrane accounted for the observed IR-induced [Ca\(^{2+}\)\(_{\text{free}}\)] increase.

\(K^+\) channels have been shown to regulate the cell cycle in irradiated tumor cells (27). Therefore, we analyzed by flow cytometry, the incorporation of the base analogue EdU by irradiated (0 or 2 Gy) T98G cells within the first 6 h after IR. Figure 4A shows the incorporated EdU in dependence on the DNA amount as defined by co-staining of the cells with PI as DNA-specific fluorescence dye. IR increased the cell populations residing in G1, S, and G2 phase in cell cycle with low EdU-specific fluorescence intensity (i.e., cells that did not incorporate EdU, Fig. 4B, top line). This points to an IR-induced G1, S, and G2–M arrest in T98G cells. In particular, IR decreased the ratios between cells in the S-phase that incorporated EdU (Shigh) and the G1low population on the one hand and between the G1low and the G2low populations on the other (Fig. 4B, bottom line) indicative of a profound inhibition of G1–S transition and mitosis in irradiated T98G cells.

To test for a function of IK channels in cell-cycle control, the effect of IR (0, 2, 4, or 6 Gy) in combination with IK inhibition by TRAM-34 on cell-cycle distribution of T98G cells was analyzed 24 and 48 hours after IR by PI staining in flow cytometry (Fig. 4C). Twenty-four hours after IR with 2 and 4 Gy, the G1 population was decreased and the S and G2 increased as compared to 0-hour values (open circles in Fig. 4D, left). This suggests that the G1 arrest observed in the EdU incorporation experiments was short-living. In contrast, 24 hours after IR with 6 Gy, the accumulation in S and G2 phase of cell cycle was blunted as compared with 2 or 4 Gy–irradiated cells suggestive of a sustained G1 arrest induced in a fraction of cells at higher dose (open circles in Fig. 4D, left). However, 48 hours after IR, number of G1 and G2 residing cells decreased and increased, respectively, more or less linearly with increasing IR dose (open circles in Fig. 4B and D, right).
confirming the transitory nature of the G₁ arrest. Importantly, the IK channel blocker TRAM-34 (10 µmol/L) delayed or even prevented the radiation-induced decrease of cell population in G₁ and accumulation in G₂ (Fig. 4D, closed triangles). Together, the data indicate functional significance of IK channels in cell-cycle control. Because only little effect of TRAM-34 on cell-cycle distribution was apparent in unirradiated cells (0 Gy in Fig. 4D), IK channel blockade does not impair the clonogenic survival of U87MG cells.

Next, we estimated the number of residual DNA double-strand breaks in T98G cells 24 hours after IR with 0 or 2 Gy by counting the γH₂AX foci in immunofluorescent micrographs (Fig. 5A). As shown in Fig. B (right), TRAM-34 (10 µmol/L) significantly increased the mean number of residual γH₂AX foci per nucleus from about 4 (vehicle control) to 6 (TRAM-34) 24 hours after IR with 2 Gy. Thereby, foci numbers seemed to be similarly elevated in nuclei with low, intermediate, and high foci counts as compared with the respective vehicle controls giving rise to a TRAM-34-induced right shift of the foci count/nucleus number histogram depicted in Fig. 5C. This right shift might be explained by a delay in DNA double-strand break repair in TRAM-34-treated cells.

Unirradiated cells showed a tendency of increased foci formation when incubated for 24 hours with TRAM-34 (Fig. 5B, left) that might hint to a genotoxic effect of TRAM-34. However, TRAM-34 (10 µmol/L) did not decrease the plating efficacy (0.25 ± 0.001, n = 36) when compared with the vehicle control (0.23 ± 0.001, n = 36) in delayed plating colony formation assays. Similarly, TRAM-34 did not decrease the plating efficacy of U87MG cells (0.56 ± 0.01 vs. 0.51 ± 0.01, n = 36), indicating that IK channel blockade does not impair the clonogenic survival of unirradiated glioblastoma cells. In irradiated T98G cells (Fig. 6A) and U87MG cells (Fig. 6B), in sharp contrast, TRAM-34 demonstrated significant decreases in clonogenic survival with radiosensitizer enhancement factors of about 1.4 (T98G) and 1.3 (U87MG) as determined for the survival fraction of 0.5. This suggests similar radiosensitizing effects of TRAM-34 in 2 human glioblastoma cell lines that differ in radiosensitivity (survival fractions at 2 Gy, SF₂Gy, of T98G and U87MG cells were SF₂Gy = 0.56 ± 0.01 and SF₂Gy = 0.74 ± 0.02, respectively; compare open bars in the insets of Fig. 6A and B).

To test whether the observed IK-mediated cell-cycle control in irradiated T98G cells might be required for DNA repair, we determined the number of residual γH₂AX foci in both T98G clones 24 hours after IR with 0 or 2 Gy. As shown in Fig. 7D and E, the IK-depleted clone #3 exhibited higher number of basal (0 Gy) and residual γH₂AX foci as compared with the control clone #2 suggestive of an impairment of DNA repair by IK knockdown. To test this assumption, the radiosensitivity of both clones was determined by delayed plating colony formation assay.

As a result, both T98G clones were more radiosensitive than the parental T98G cell line (compare open circles in Fig. 7F and Fig. 6A). Notably, the IK-depleted clone #3 was significantly more radiosensitive (SF₂Gy = 0.74 ± 0.03, n = 12) than the control clone #2 (SF₂Gy = 0.83 ± 0.02, n = 12; P = 0.02, Welch-corrected two-tailed t test). Most importantly, TRAM-34 radiosensitized only the control clone #2 (Fig. 7F, left) but, again, had no effect on the IK-depleted clone #3 (Fig. 7F, right). Combined, these data indicate both IK-mediated radiosensitivity in human glioblastoma cell lines and target specificity of the IK channel blocker TRAM-34.

To test whether IK channel targeting may increase the efficacy of fractionated radiation in an in vivo ectopic glioblastoma mouse model, immunocompromised nude mice were challenged with
human U87MG glioblastoma cells. When the ectopic glioblastoma has reached a volume of around 150 μL (Fig. 8A), mice were allocated to four treatment arms [control, n = 5; TRAM-34, n = 4; fractionated IR (fIR), n = 9; and TRAM-34/IR, n = 6]. Figure 8B and C shows the tumor volume (V_t) normalized to the respective tumor volume at the start of treatment on day 0 (V_0). before, during (arrows), and after treatment with fIR (5 × 0 or 5 × 4 Gy) and TRAM-34 injections (5 × 0 or 5 × 120 mg/kg body weight) 6 hours prior to each IR fraction. One of six mice treated with combined fIR/TRAM-34 and 2 of 9 mice treated with fIR alone showed complete tumor remission. One of the latter did even not progress during treatment and could not be included in the calculation of the time to progression (i.e., the period between treatment start on day 0 and the time when the treated glioblastomas exceeded the initial volume, V_0). This time to progression is given for all treatment groups in Fig. 8 demonstrating that only the IR/TRAM-34 group exhibited significantly longer time-to-progression periods than the control group.

The exponential growth of the ectopic glioblastoma can be illustrated by the linear relationship between the mean (±SE) logarithmized tumor volume [ln(V_t/V_0)] and the time as depicted for the four treatment groups in Fig. 8E and F. The slope of these relationships [δ[ln(V_t/V_0)/δt]] as a measure of the exponential growth kinetics before and during the treatment as well as the treatment-induced slope decline [Δ[ln(V_t/V_0)/δt]] are given for the individual tumors in all four treatment groups in Fig. 8G–I. Only the fIR/TRAM-34 group showed a significant treatment-induced decrease in exponential growth as compared with the control group (Fig. 8f). Together, these in vivo experiments suggest both that TRAM-34 can be applied at pharmacologically relevant doses and that concomitant TRAM-34 chemotherapy may increase the efficacy of fractionated radiation therapy in vivo.

To explore the potential function of IK channels for the glioblastoma therapy resistance observed in the clinic, TCGA was queried using the provisional open access Glioblastoma-Multiforme and Lower-Grade-Glioma databases. As shown in Fig. 9, high IK mRNA abundance is associated with a shorter PFS (Fig. 9A) and OS (Fig. 9B) of patients with lower grade glioma and shorter OS (but not PFS, Fig. 9C) of patients with glioblastoma (Fig. 9D).

**Discussion**

The present study demonstrates IR-induced Ca^{2+}-signaling and activation of Ca^{2+}-activated intermediate conductance IK K^+ channels in glioblastoma cells. The IR-stimulated IK channels, in turn, contribute to the stress response of the glioblastoma cells probably by adjusting the cell cycle. This IK channel-mediated stress response is required for the survival of the irradiated glioblastoma cells as evident from the fact that pharmacologic blockade of the IK channels radiosensitized the glioblastoma cells. In an astrocyte cell line, in contrast, functional IK channels probably by adjusting the cell cycle. This IK channel-mediated stress response is required for the survival of the irradiated glioblastoma cells as evident from the fact that pharmacologic blockade of the IK channels radiosensitized the glioblastoma cells. In an astrocyte cell line, in contrast, functional IK channels were not apparent.
IR-induced modifications of Ca²⁺ signaling and/or K⁺ channel activity have been reported by our group in different tumor entities such as lung adenocarcinoma (28), leukemia cells (27, 29), or glioblastoma (21). In lung adenocarcinoma, K⁺ channels contribute to an elevated glucose uptake by the irradiated cells. Increased amounts of glucose are probably needed to counteract energy crisis caused by DNA damage and to provide the carbohydrates required for histone acetylation during DNA decondensation (30). In leukemia, IR-induced co-activation of both Ca²⁺-permeable channels and K⁺ channels gives rise to Ca²⁺ signals that induce cell-cycle arrest via CaMKII-mediated inhibition of the mitosis-promoting factor cdc2. Notably, pharmacologic K⁺ channel blockade overrides cell-cycle arrest of irradiated leukemia cells resulting in radiosensitization (27).

In glioblastoma, IR-induced activation of BK K⁺ channels is associated with radiogenic hypermigration of the tumor cells (for review, see refs. 15, 16). Like BK, IK channels have been demonstrated to essentially contribute to the mechanics of serum-induced (34), bradykinin-induced (6), and CXCL12 (SDF-1)-induced glioblastoma cell migration (35). In accordance with these in vitro data is the observation that the IK inhibitor TRAM-34 blocks the brain infiltration by xenografted human glioblastoma cells in orthotopic mouse models (36).

High IK channel expression has been associated with upregulation of “stemness” markers (8), and the glioblastoma “stem” cells have been suggested to express a highly migratory phenotype and to be primarily responsible for brain invasion (37, 38). As a matter of fact, IK channels have been demonstrated to mediate the migration of neuronal precursor cells, so-called neuroblasts.
along the rostral migratory stream to become interneurons in the olfactory bulb of normal adult mouse brain (39).

Glioblastoma 'stem' cells are also thought to be more therapy-resistant than the bulk tumor mass of "differentiated" glioblastoma cells (for review, see ref. 15–17). The data of the present study on glioblastoma cell lines and on an ectopic mouse model suggest that IK channels may confer radioresistance besides promoting brain infiltration. Evidence for such an IK channel function in glioblastoma cells obtained in vitro has already been reported (20).

The potential dual function of IK channels for brain invasion and radioresistance of glioblastoma as suggested by the above-mentioned in vitro and animal studies might be reflected by recently reported retrospective clinical data. Querying the REMBRANDT patient gene data base of the National Cancer Institute has indicated an upregulation (1.5-fold greater than nontumor samples) of IK channel in more than 30% of the patients (10). Importantly, IK upregulation by the glioma correlates with a decreased survival of the patients (10). Likewise, querying the TCGA databases in the present study suggested that higher IK mRNA abundance in the glioma associates with shorter PFS (low-grade glioma) and OS (low-grade glioma and GBM) of patients with glioma. Subgroup analysis of the patients concerning, for example, tumor size, degree of surgical glioma resection, radiation therapy regimes, etc., could not be performed in the TCGA query and has not been reported in the REMBRANDT query (10), which limits the interpretation of the data. Nevertheless, provided that many patients of the databases received therapy regimes that comprise radiation therapy, the found associations might hint to a radioprotective function of IK channels in glioma.

IK channels might, therefore, become a highly attractive new target for anti-glioma therapy. IK channel targeting has been proposed for therapy of different diseases such as anemia (40, 41), in particular sickle cell anemia (42–45), Alzheimer disease (46), and various further inflammatory diseases (47). The TRAM-34 concentration (1–10 mmol/L) used in the present study is probably far above the plasma concentrations that might be reached in clinical trials. Senicapoc (ICA-17043), a further IK channel inhibitor, which is more potent than TRAM-34 (IC50-Senicapoc, 1 nM vs. IC50-TRAM-34, 20 nmol/L), can be taken orally and has been shown to be safe in clinical trials (46). Moreover, a daily oral dose of 10-mg senicapoc resulted in mean plasma concentrations of 100 ng/mL (~0.3 μmol/L). Most importantly, senicapoc-containing plasma samples of the patients inhibited IK channels by up to 70% as assessed in tracer flux experiments (43). What is also important in this respect is the fact that GBM reportedly impairs the blood–brain barrier (BBB) by, for example, altering/replacing endothelial cells (48) and pericytes (49), suggesting that drugs like senicapoc or TRAM-34 may pass the BBB. In a mouse brain, a BBB passage of TRAM-34 could be directly demonstrated (36). Taken together, these data indicate that IK channel targeting is most probably feasible in a clinical setting. Higher drug levels at lower side effects might even be achieved in patients with glioblastoma by intracranial drug administration.
In conclusion, IK channels may promote beside a migratory and infiltrative phenotype also cellular radioresistance of glioblastoma cells. By doing so, IK channels contribute to those properties of GBM that most probably account for therapy failure associated with the very poor prognosis of patients. Importantly, pharmacologic IK channel targeting seems to be feasible in the clinic in combination with surgery and radiation therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: B. Stegen, L. Butz, S.M. Huber

Development of methodology: B. Stegen, L. Butz, L. Klumpp, K. Dittmann, S.M. Huber

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Stegen, L. Butz, L. Klumpp, S.M. Huber

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Stegen, L. Butz, D. Zips, P. Ruth, S.M. Huber

Writing, review, and/or revision of the manuscript: B. Stegen, L. Klumpp, D. Zips, S.M. Huber

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Stegen, L. Butz, S.M. Huber

Study supervision: P. Ruth, S.M. Huber

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Benjamin Stegen, Lena Butz, Lukas Klumpp, et al.


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