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Increased cytotoxicity of ionizing radiation in combination with membrane-targeted apoptosis modulators involves downregulation of protein kinase B/Akt-mediated survival-signaling

René Handrick[a,1], Amelie Rübel[a,1], Heidrun Faltin[a], Hansjörg Eibl[b], Claus Belka[a], Verena Jendrossek[a,*]

[a]*Department of Radiation Oncology, Experimental Radiation Oncology, University of Tübingen, Germany,* [b]*Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany*

Abstract

***Background and purpose*: The membrane-targeted apoptosis modulators erucylphosphocholine (ErPC) and erucylphosphohomocholine (ErPC3) induce apoptosis in highly apoptosis resistant malignant glioma cell lines and enhance radiation-induced cell death and eradication of clonogenic tumor cells in vitro. Aim of the present study was to elucidate molecular mechanisms of combined action.**

***Materials and methods*: Induction of apoptosis was evaluated by determination of nuclear morphology (fluorescence microscopy), alteration of mitochondrial function and caspase-activation (flow cytometry, Western blot). Activity of protein kinase B (PKB/Akt) and key downstream effectors involved in apoptosis regulation was verified by Western blot analysis using activation-specific antibodies.**

***Results*: Increased cytotoxicity of the combination was linked to a more efficient activation of the intrinsic apoptosis pathway with increased damage of the mitochondria and caspase-activation. Moreover, activity of the survival kinase PKB/Akt was downregulated upon treatment with ErPC/ErPC3 alone or in combination with ionizing radiation. Inhibition of PKB/Akt was associated with decreased phosphorylation and thus activation of the pro-apoptotic Bcl-2 protein Bad as well as dephosphorylation of the transcription factor FOXO3A (FKHRL1) that may be responsible for the observed increased expression of the pro-apoptotic Bcl-2 protein Bim.**

***Conclusions*: Our data suggest a role for inhibition of PKB/Akt-mediated anti-apoptotic signaling in increased efficacy of the combination.**

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During the last decades there has only been little progress in the therapy of patients suffering from malignant glioma. Even the combined use of refined surgery, improved technology of the application of radiation therapy (RT) and aggressive chemotherapy failed to significantly improve survival in particular in patients suffering from the most aggressive manifestation of malignant glioma, glioblastoma multiforme (GBM) [1,4,28]. Due to the high intrinsic resistance of malignant glioma cells against DNA-damaging drugs and the failure of many chemotherapeutic agents to cross the blood—brain barrier, up to now only few drugs proved to be active against malignant glioma in clinical settings including classical alkylating nitrosurea-derivatives, temozolomide and irinotecan [17,35,36]. Therefore, novel agents that efficiently penetrate into the brain and that are active against highly resistant malignant glioma are urgently needed.

Unfortunately, malignant glioma cells are mostly characterized by genetic alterations in tumor suppressor genes and/or oncogenes involved in cell cycle control, activation of proliferation and inhibition of apoptosis. On the one hand, loss or mutation of the tumor suppressor p53 increased expression of the p53-antagonist mdm2 or increased expression of the anti-apoptotic protein Bcl-2 can contribute to apoptosis resistance [16,26]. On the other hand, deregulated activity of the phosphatidyl-inositol-3-kinase (PI3K)/PKB/Akt pathway which is crucial for the propagation of survival signals originating from growth factor

[1] Both authors contributed equally to this work.

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receptor tyrosine kinases may allow the tumor cells to escape from cell death through inhibition or downregulation of pro-apoptotic proteins of the Bcl-2 family [13,26,33,34,39].

Since aberrant apoptosis and survival pathways can contribute to treatment resistance of malignant glioma they constitute an attractive target for the modulation of the therapy response. In this regard, antineoplastic alkylphosphocholines represent a novel class of antineoplastic agents that target cellular membranes and potently induce apoptosis in a variety of human solid tumor cells including malignant glioma cell lines without direct interaction with the DNA. Although similar to ionizing radiation these drugs induce apoptosis via the mitochondrial death pathway they can increase cytotoxicity of ionizing radiation [19,29,30,38]. However, up to now only single derivatives have been identified that may be suited for clinical application in particular for the treatment of malignant glioma. Of these, the first intravenously applicable alkylphosphocholine ErPC can cross the blood–brain barrier of healthy rats upon repeated intravenous injections and accumulate in brain and brain tumor tissue [7,8]. Moreover, ErPC and its structural derivative ErPC3 (Erufosine) efficiently induce growth arrest and apoptosis in highly resistant human astrocytoma/glioblastoma cell lines in vitro [18,20].

Most importantly, ErPC and ErPC3 increased the radiation response of human malignant glioma cell lines in vitro. Both drugs enhanced the extent of radiation-induced cell death in short time assays and increased eradication of clonogenic tumor cells in long term colony formation assays. Mechanistic investigations suggest an involvement of increased cell death induction for improved efficacy of the combination [29].

Aim of the present investigation was to characterize the molecular mechanisms of increased cytotoxicity of the combination in more detail with a focus on apoptosis induction and regulation of apoptosis sensitivity by the survival kinase PKB/Akt. Our data demonstrate that ErPC and ErPC3 efficiently enhance radiation-induced apoptosis in malignant glioma cell lines and implicate a critical role of down-regulated PKB/Akt-mediated survival signaling in increased apoptosis rates upon combined treatment.

Methods

Chemicals and drugs

ErPC and ErPC3 were synthesized by H. Eibl, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany, and dissolved to a final concentration of 10 mM (stock solution) as described elsewhere [29]. Hoechst33342 (Calbiochem, Bad Soden, Germany) and propidium iodide (PI) were dissolved in distilled water as 1.5 mM or 5 mg/ml stock solution, respectively. zVAD-fmk was from Bachem Distribution services GmbH (Hamburg, Germany).

Rabbit antibodies against full length and cleaved PARP, full length and cleaved caspase-3, anti-Akt, anti-phospho(p)-Akt (Ser473), anti-p-Akt (Thr308), anti-Bad, anti-p-Bad (Ser136), anti-FOXO3A, anti-p-F3A, anti-Bax and anti-Bak were from Cell Signaling (Frankfurt, Germany). Mouse anti-human Bcl-X_L clone 7B2.5 recognizing Bcl-X_L and Bcl-X_S was obtained from Upstate (Biomol, Hamburg, Germany). Anti-Bim was purchased from Pharmingen (Becton–Dickinson, Heidelberg, Germany). Anti-caspase-9 and activation specific Anti-BaxNT antibody were obtained from Upstate (Biomol, Hamburg, Germany). Mouse anti-β-actin (antibody) was from Sigma. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were from Amersham-Biosciences (Freiburg, Germany).

All other chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany) if not otherwise specified.

Cell lines, cell culture and cellular treatment

T98G and A172 astrocytoma/glioblastoma cell lines were from ATCC (Bethesda, MD, USA). For all experiments cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Gibco Life Technologies, Eggenstein, Germany) and maintained in a humidified incubator at 37 °C and 5% CO_2.

Irradiation was performed at room temperature with 6 MV photons from Siemens or Elekta linear accelerators with a dose rate of 2 or 4 Gy per min, respectively. ErPC or ErPC3 was added to the culture medium immediately after irradiation. Pretreatment with zVAD-fmk was performed for 30 min prior to irradiation and/or ErPC-treatment.

Determination of cell death and caspase-activation

Cell death was analyzed by fluorescence microscopy (Zeiss Axiovert 200, Carl Zeiss, Jena, Germany) upon combined staining of the cells for 10 min with Hoechst33342 and PI at a final concentration of 1.5 μM and 2.5 μg/ml, respectively, using a G365/FT395/LP420 filter set at 40× magnification. Apoptotic cells and necrotic cells were quantified by cell counting [29]. Caspase-activation was determined by Western blot analysis of cytosolic extracts as described below using antibodies against the caspase-substrate PARP as well as against active cleavage fragments of caspases-9 and -3.

Bax-activation

Activation of Bax was quantified by flow cytometry using an activation specific antibody. In brief cells were washed with PBS and permeabilized in 200 μl permeabilization buffer (0.1% v/v Triton/PBS) for 10 min at RT. After washing in PBS 200 μl blocking buffer (10% v/v FCS/PBS) were added for 15 min. Following a wash step cells were incubated in 50 μl anti Bax-NT rabbit polyclonal IgG (0.1 μg/1 × 10^5 cells, 10% FCS/PBS). After washing in 200 μl blocking buffer cells were incubated with Alexa Fluor488-conjugated anti-rabbit antibody (Molecular Probes, Mobitech, Goettingen, Germany) in 10% FCS/PBS for 30 min at 4 °C in the dark. Finally, cells were washed in 200 μl blocking buffer resuspended in 100 μl PBS and fluorescence-shift (geo mean) was quantified at FL-1 in FACS Calibur (BD, Heidelberg, Germany).

Western blot analysis

Cell lysates were performed as described elsewhere [29]. Twenty micrograms of lysate were separated by SDS–PAGE and blotted onto PVDF-membranes (Roth, Karlsruhe, Germany). After blocking membranes were incubated overnight

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