A chemical screen for medulloblastoma identifies quercetin as a putative radiosensitizer

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ABSTRACT

Purpose
Treatment of medulloblastoma in children fails in approximately 30% of patients, and is often accompanied by severe late sequelae. Therefore, more effective drugs are needed that spare normal tissue and diminish long-term side effects. Since radiotherapy plays a pivotal role in the treatment of medulloblastoma, we set out to identify novel drugs that could potentiate the effect of ionizing radiation selectively.

Experimental design
A small molecule library, consisting of 960 chemical compounds, was screened for its ability to sensitize towards irradiation, using two independent read-out systems.

Results
This small molecule screen identified the flavonoid quercetin as a novel radiosensitizer and we show that quercetin can sensitize the medulloblastoma cell lines DAOY, D283-med, and, to a lesser extent, D458-med to ionizing radiation at low micromolar concentrations. Quercetin enhanced the in vitro radiation sensitivity of medulloblastoma cells as determined with clonogenic survival assays at doses used in fractionated radiation schemes. Furthermore, quercetin did not affect the proliferation of neural precursor cells or normal human fibroblasts. DAOY and D283-med were identified as a SHH and a group 3 subtype medulloblastoma, respectively, which may indicate a subgroup-independent effect of quercetin on the radiation response. Importantly, in vivo experiments confirmed the radiosensitizing properties of quercetin, as administration of this flavonoid at the time of irradiation significantly prolonged survival in orthotopically xenografted mice.

Conclusion
Together, these findings indicate that quercetin is a potent radiosensitizer for medulloblastoma cells that may be a promising lead for the treatment of medulloblastoma in patients.
INTRODUCTION

Medulloblastoma, the most common malignant brain tumor in children, accounts for approximately 20% of all intracranial childhood tumors. Medulloblastomas are invasive neuroepithelial tumors that are thought to arise from the progenitor cells of the dorsal brain stem, or from precursor cells in the cerebellum. Currently, patients are stratified into two groups based on histological features and clinical criteria: the standard- and high-risk groups. Treatment of standard-risk medulloblastoma patients consists of surgery and reduced-dose craniospinal radiotherapy, followed by an additional boost aimed at the primary tumor site, and adjuvant chemotherapy. In high-risk patients, a higher craniospinal dose is used. Although such treatment has resulted in remarkable improvement in outcome, this therapy still fails in approximately 30% of medulloblastoma patients. Moreover, such therapy causes severe long-term side effects that significantly impact quality of life. There is a need for alternative therapies that allow to lower the total dose of irradiation or increase the radiation efficacy. Besides new radiotherapy techniques that limit the radiation doses to surrounding healthy tissues, new chemotherapeutics that specifically sensitize the tumor to irradiation may provide alternative therapies.

We screened a commercially available small molecule library consisting of 960 compounds (ActiTarg-K960) with a diversity of chemical structures to identify radiosensitizers for medulloblastoma. Chemical structures represented by these compounds have been reported to exert kinase inhibitory functions and screening of these compounds would help in the identification of new chemical directions for hit optimization. However, to our surprise, we identified a known chemical compound, quercetin (3,3',4',5,7-pentahydroxyflavone), a flavonoid found in fruits, vegetables and grains, as a radiosensitizer for human medulloblastoma cells. Quercetin treatment at low micromolar concentrations did not affect cell proliferation when used as monotherapy, while the combination with irradiation significantly decreased medulloblastoma cell growth. Importantly, this sensitizing effect was not found on neural precursor cells, or normal human fibroblasts. In addition, quercetin treatment enhanced the in vitro sensitivity of medulloblastoma cell lines in clonogenic survival assays. However, the radiosensitizing effect was not observed in two primary medulloblastoma cell cultures. Finally, we observed that quercetin administration to orthotopically xenograft mice around the time of irradiation significantly prolonged survival. Since quercetin sensitizes medulloblastoma cells in our experiments at doses used in fractionated radiation schemes, and the quercetin concentrations used can easily be achieved by oral administration, we suggest that the use of quercetin should be further evaluated in clinical trials in medulloblastoma patients in the near future.
MATERIALS AND METHODS

Cell culture and lentiviral infections

Mouse C17.2 neural precursor cells, human primary fibroblasts and D283-Med, D458-Med, DAOY medulloblastoma cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere in DMEM plus 10% fetal calf serum, 100IU/ml penicillin and 100mg/ml streptomycin (PAA Laboratories GmbH, Austria). Pools of D283-med FM/GC or DAOY FM/GC cells were generated by infection with lentiviral vectors, expressing the reporter gene combinations Firefly luciferase/mCherry (FM) or Gaussia luciferase/Cerulean (GC), as described previously. Fluorescence microscopy was used to assess the success rate of transductions and cell viability. For intracranial injections, cells were harvested and suspended in PBS at a concentration of 1x10⁸ cells/ml. The cells used in this study were not authenticated.

Primary cell culture VU371 was derived from tumor tissue, surgically removed from a patient diagnosed with medulloblastoma at the VU University Medical Center. Informed consent was obtained according to institutionally approved protocols. ICb-1299MB is a patient derived orthotopic xenograft mouse model of group 4 medulloblastoma and the xenograft cells were kindly provided by Dr. Xiao-Nan Li and Dr. Mari Kogiso (Baylor College of Medicine, Houston, TX, USA). VU371 and ICb-1299MB were cultured at 37°C in a 5% CO₂ humidified atmosphere in NBM (NeuroBasal Medium)(Invitrogen) supplemented with neural stem cell supplement (NSCS), N2, stable glutamine (PAA), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF)(PeproTech).

Chemicals

The ActiTarg-K960 chemical library, consisting of 960 putative kinase inhibitory compounds, was obtained from TimTec (Newark, Delaware, USA). Ninety-one percent of these compounds conform to four Lipinski criteria and 97% to three Lipinski criteria, suggesting they have desirable pharmacologic properties. Quercetin dihydrate (Calbiochem, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), with a final DMSO concentration of 0.1% for in vitro experiments or a final DMSO concentration of 5% for in vivo experiments.

Chemical library screen

Seven hundred and fifty DAOY cells were plated per well in 96-well plates. The next day, cells in each well were treated with a different compound from the ActiTarg-K960 library at a 1 µM concentration. Treatment was performed with drugs in paired 96-well plates, where one plate was exposed to 4 Gy in a Gammacell® 220 Research Irradiator (MDS Nordion, Canada) 30 minutes after addition of the compounds, and the other plate was a non-irradiated control. Four days later, cell survival was evaluated by measuring Gaussia luciferase (Gluc) activity, or by means of the Acumen X3 laser scanning
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Survival assays
Responsiveness of D283-med, D458-med (1,000 cells/well, or 1x10^4 cells/flask), or DAOY (1,500 cells/well) medulloblastoma cells to quercetin (1 µM) was determined in a cell proliferation assay by use of the Acumen +X3 laser scanning cytometer as described above, and confirmed by cell counts using a Bürker hemocytometer. Viability of primary cells, VU371 and ICb-1299MB (2,000 cells/well), after treatment with 1 µM quercetin and 0.7 Gy irradiation was determined with Cell Titer Glo assay (Promega) according to manufacturer’s protocol. In addition, a clonogenic assay was performed with different doses of irradiation (0-3 Gy). Therefore, exponentially growing D283-med and D458-med cells were plated in triplicate in 6-well plates at concentrations ranging from 200-2,000 cells/well, and grown for 14 days in MethoCult®H4001 with 0, 0.5, or 1 µM quercetin. Colonies were counted visually and plating efficiency (PE) was calculated by dividing the number of colonies counted by the number of cells plated. Surviving fractions (SF) were then calculated by dividing the PE by the PE of the non-irradiated control per drug concentration. Duplicate experiments were performed for each cell line. Analysis of inhibitory concentrations was performed using SigmaPlot 11.0 (Systat Software, Inc. San Jose, CA, USA).

qRT-PCR
Quantitative RT-PCR (qRT-PCR) analysis was performed to determine expression of the medulloblastoma subgroup classifiers WIF1, SFRP1, NPR3, and KCNA in medulloblastoma cell lines and a normal cerebellum sample. Total RNA was isolated using the TRizol RNA isolation protocol (Invitrogen, Carlsbad, CA, USA) and equal amounts of RNA were converted to cDNA using the Omniscript kit (Qiagen). Primer sequences for WIF1, SFRP1, NPR3, and KCNA transcripts were previously described by Zhao et al.20 and primers were manufactured by Biolegio (Nijmegen, The Netherlands). Gene expression of the subgroup classifiers in medulloblastoma cells was normalized to GAPDH expression levels and the Ct values were used to calculate the relative fold difference in mRNA levels (ΔΔCt method) compared to normal cerebellum.

D283-med orthotopic xenograft mouse model
Female athymic nude-Fox1nu mice (age 8-10 weeks; Harlan, Horst, The Netherlands) were maintained in accordance with the guidelines and regulations set out by the VU University committee on research animal care. For intracranial injections, mice were anesthetized with 2.5% isoflurane in oxygen, and a volume of 5 µl (0.5x10^5 D283-med FM-GC cells) was injected stereotactically into the cerebellum at a rate of 2 µl per minute, using a Hamilton 10 µl syringe with a 26G needle. Coordinates for injection were determined according to the mouse brain atlas23: 2.0 mm lateral, 2.5 mm ventral
of lambda, and at a 2.0 mm depth. Before start of the treatment (three weeks after injection of the tumor cells) tumor engraftment was determined by measuring Firefly luciferase (Fluc) activity. Based on this activity, mice were randomized into four treatment protocols: vehicle (5% DMSO), quercetin, DMSO/irradiation (4 Gy), and quercetin/irradiation (4 Gy). Mice without tumor engraftment (Fluc activity <80,000 photons/sec at three weeks after implantation) were excluded from the experiment. Quercetin (100 mg/kg) was administered intraperitoneally at six time points: 30 and 60 minutes before-, or after irradiation, 0 hours, and 24 hours after irradiation. Mice not receiving quercetin were treated with the same volume of DMSO dissolved in PBS, at the same time intervals as the quercetin treated mice. To enable precise positioning of the radiation beam on the head and neck area of the mice, the animals were anesthetized with ketamin/xylazin. Mice that were not irradiated were anesthetized just before the zero hours’ time point. Tumor growth was monitored semi-weekly by bioluminescent imaging (BLI). In short, 150 µl D-luciferin (0.03 g/L, Gold Biotechnology, St. Louis, USA) was injected intraperitoneally and 10 minutes after administration mice were anesthetized with isoflurane inhalation anaesthesia, positioned in the IVIS camera and the bioluminescence signal was determined with the IVIS Lumina CCD camera. In addition, the mice were monitored daily for discomfort and weight loss. When moderate to severe symptoms were present (weight loss of >20% or severe neurological deficits), animals were sacrificed and brains were removed and formalin-fixed.

Statistical analysis
A coefficient of variation (CV) and Z’ factor were calculated to assess the reproducibility and robustness of the small molecule screens, as described by Zhang et al., where CV=SD/µ and Z’ = 1-(3σc+ +3σc−)/|µc+ −µc−|. Statistical significance of treatment was assessed using the Mann-Whitney U test. Kaplan-Meier survival curves were generated with GraphPad Prism 5. Median survival of the groups was calculated and survival curves were compared with the Log-rank (Mantel-Cox) test. The p values <0.05 were considered statistically significant.

RESULTS
Identification of quercetin as a radiosensitizer for medulloblastoma
In order to enable the identification of novel radiosensitizers for medulloblastoma, a small molecule screen was performed using DAOY medulloblastoma cells that were transduced with a lentiviral Gaussia luciferase (Gluc) vector co-expressing the fluorescent ‘Cerulean’ (CFP) reporter. Expression of these genes allowed to monitor cell survival by bioluminescent and fluorescent read-out of cell viability. To optimize screening conditions, the well-to-well and plate-to-plate variation, number of DAOY cells, and the dose of irradiation were determined. For the well-to-well and plate-to-plate variation, aliquots of Gluc-conditioned medium were distributed over 96-well
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plates and assayed for luciferase activity as described previously\(^\text{22}\). In four independent experiments a variation coefficient (CV) of < 7% was observed (Fig. 1A), indicating only minimal variation in pipetting errors, substrate stability and measurement errors. An even better CV of < 2% was observed (Fig. 1A) for the plates measured by Acumen technology, where equal numbers of cells were plated and detected by CFP expression. Robustness of the assays was determined by calculating the Z’ factor as described by Zhang \textit{et al.}\(^\text{24}\), where Z’=0.84 for the Gluc assay and Z’=0.56 for the Acumen screen. Since both assays allowed to monitor cell viability at different time points after treatment, we optimized our screening conditions – number of cells, dose of irradiation, and drug concentrations – by measuring Gluc secretion or cell numbers in time (Fig. 1B-D). This resulted in a four-day assay, using 750 DAOY cells per well with 4 Gy irradiation. In addition, a drug concentration of 1 µM was chosen, since this showed good results in a pilot experiment using eight different, randomly chosen small molecules (Fig. 1D), and yielded positive hits in a drug screen performed previously by our group\(^\text{22}\). Once all conditions were established, cells were seeded in 96-well plates, and treated the next day with compounds from the ActiTarg-K960 drug library (TimTec) consisting of 960 putative kinase inhibitors, or with 0.1% DMSO as an internal control. The compounds were added in duplicate plates, where one set was irradiated to identify putative radiosensitizers, while the other set was left non-irradiated to assess cytotoxicity of the compounds as monotherapy. A reduction of >75% of cell growth after four days of incubation as compared to the DMSO controls was considered to be significant (Fig. 2A). In four separate screens, a total of 23 compounds were identified that consistently inhibited cell growth or sensitized towards irradiation, with 12 compounds inducing cell death independently of irradiation, and 11 compounds functioning as radiosensitizers (Table 1 and Supplementary Fig. S1). Cytotoxicity of these 23 compounds was subsequently determined on primary human fibroblasts and on C17.2 neuronal precursor cells (NPCs), to assess the therapeutic window (Table 1). This smaller screen narrowed our list of putative novel compounds for use in medulloblastoma down to five: two radiosensitizing agents and three compounds that have been identified as inducers of cell death in DAOY cells independently of irradiation (Fig. 2B). The flavonoid quercetin was among the radiosensitizing compounds that has been shown to enhance radiation-induced cell death in rat hepatoma cells\(^\text{25,26}\). Treatment with quercetin 30 minutes prior to irradiation resulted in a 5-fold reduction in cell growth (~20% cell survival), while treatment with quercetin alone did not significantly affect cell viability compared to cells treated with the solvent DMSO (Fig. 2C). Irradiation without addition of quercetin resulted in a 2-fold reduction in cell numbers. As mentioned above, these results were not observed in primary human fibroblasts or neuronal precursor cells (Fig. 2C).

\textbf{Quercetin sensitizes medulloblastoma cells to radiation in vitro}

Since quercetin has been reported to effectively cross the blood-brain-barrier\(^\text{27}\), we hypothesized that this compound could be an attractive agent for the treatment of
Figure 1. Determination of screening conditions. (A) Reproducibility of Gluc measurements (upper panel) or cell counts as measured by Acumen technology (lower panel). Aliquots of Gluc-containing medium or equal numbers of cells were plated in 96-well plates in quadruplicate, and measured to assess plate-to-plate and well-to-well variation (RLU=relative luciferase units). Data are presented as means ± SD. The corresponding coefficients of variation (CV) are depicted in the right-hand panels. (B) Growth curves of DAOY medulloblastoma cells. One hundred, 250, 500, 750, or 1000 cells were plated per well and set at 100%. Relative cell numbers were measured at different time points after plating as indicated in the figure. A plating density of 750 cells/well resulted in exponentially growing cells after 4 days of incubation that could be monitored without much variation. Data are presented as means ± SD (n=3). (C) Graphic representation of the irradiation response in DAOY cells. Growth of non-irradiated (NI) cells is set at 100%. For each irradiation dose 8 samples were measured. (D) Concentration curves of DAOY medulloblastoma cells treated for four days with 0, 0.5, 1, or 2 µM of different drugs. Compounds were chosen randomly from the TimTec library: ST027883 (-■-), ST004727 (-●-), ST053862 (-▲-), ST012157 (-▼-), ST012256 (-♦-), ST029265 (-○-), ST036501 (-□-), ST052055 (-△-). A drug concentration of 1 µM was (red dotted line) used for the final screen.
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### Table 1. Overview of compounds that induce cell death in DAOY medulloblastoma cells, as identified by a small molecule screen.

<table>
<thead>
<tr>
<th>IUPAC name</th>
<th>Structure formula</th>
<th>Molecular weight (Da)</th>
<th>Common name</th>
<th>Fibroblasts</th>
<th>Neural Precursor Cells</th>
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<tr>
<td>(4-methoxyphenyl)(2-methylbenzo[h]quinolin-4-yl)amine, chloride</td>
<td>C21H19CN2O</td>
<td>350,85</td>
<td>OK</td>
<td>OK</td>
<td></td>
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<tr>
<td>6-amino-4-(9-ethylcarbazol-3-yl)-3-methyl-4H-pyran[3,2-d]pyrazole-5-carbonitrile</td>
<td>C22H19N5O</td>
<td>369,43</td>
<td>OK</td>
<td>OK</td>
<td></td>
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<tr>
<td>3-amino-1-[4-fluorophenyl]-1H-benzo[f]chromene-2-carbonitrile</td>
<td>C20H13FN2O</td>
<td>316,33</td>
<td>OK</td>
<td>OK</td>
<td></td>
</tr>
<tr>
<td>5-bromo-4-(4-cyclohexyl-5-phenyl(1,2,4-triazol-3-ythio))-2-phenyl-2-hydropyridazine-3-one</td>
<td>C24H22BrN5OS</td>
<td>508,44</td>
<td>OK</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>4,5-dichloro-2-[4-fluorophenyl][methyl]-2-hydropyridazine-3-one</td>
<td>C11H7Cl2FN2O</td>
<td>273,09</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>3-[([1E]-2-(2-pyridyl)-1-azavinyl)amino]-6-methyl-4H-1,2,4-triazin-5-one</td>
<td>C10H10N6O</td>
<td>230,23</td>
<td>+/-</td>
<td>cell death</td>
<td></td>
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<tr>
<td>3-[([1E]-2-(2-pyridyl)-1-azavinyl)amino]-4H-1,2,4-triazin-5-one</td>
<td>C9H8N6O</td>
<td>216,2</td>
<td>+/-</td>
<td>cell death</td>
<td></td>
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<tr>
<td>1-cyclohexylazoline-2,5-dione</td>
<td>C10H13N2O</td>
<td>179,22</td>
<td>N-cyclohexylmaleamide</td>
<td>cell death</td>
<td>cell death</td>
</tr>
<tr>
<td>4,7-dimethylpyridino[3,2-h]quinoine, oxamethane</td>
<td>C14H14N2O</td>
<td>226,28</td>
<td>neocuproine</td>
<td>cell death</td>
<td>cell death</td>
</tr>
<tr>
<td>2,9-dimethylpyridino[3,2-h]quinoine</td>
<td>C14H12N2</td>
<td>208,26</td>
<td>neocuproine</td>
<td>cell death</td>
<td>cell death</td>
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<td>5-([2E]-5,5-dichloropenta-2,4-dienyl)-6-methylpyran-2-one</td>
<td>C11H8Cl2O3</td>
<td>259,09</td>
<td>cell death</td>
<td>cell death</td>
<td></td>
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<tr>
<td>(8-chloro[4H-benzo[e]1,3-thiazolo[5,4-c]thiin-2-yl])naphthylamine</td>
<td>C20H13N2S2</td>
<td>380,92</td>
<td>cell death</td>
<td>cell death</td>
<td></td>
</tr>
<tr>
<td>2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one</td>
<td>C15H10O7</td>
<td>302,24</td>
<td>quercetin</td>
<td>OK</td>
<td>OK</td>
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<tr>
<td>3-[[tert-butyl]amino]-1-[5-methyl-2,3-diphenylindolyl]propan-2-ol</td>
<td>C28H32N2O</td>
<td>412,57</td>
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<td>OK</td>
<td></td>
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<tr>
<td>5-(4,6-dimethylpyrimidin-2-ylthio)-4-nitrobenzo[c]1,2,5-thiadiazole</td>
<td>C12H9N5O2S2</td>
<td>319,37</td>
<td>+/-</td>
<td>OK</td>
<td></td>
</tr>
<tr>
<td>3-(indol-3-ylmethylene)benzo[b]pyran-2,4-dione</td>
<td>C18H11N03</td>
<td>289,29</td>
<td>+/-</td>
<td>cell death</td>
<td></td>
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<tr>
<td>5-([1E]-2-[4-bromo-3-chlorophenyl]-2-azavinyl)-2-nitrothiophene</td>
<td>C11H6BrCN2O2S</td>
<td>345,6</td>
<td>+/-</td>
<td>cell death</td>
<td></td>
</tr>
<tr>
<td>5-([1E]-2-[2,4-dichlorophenyl]-2-azavinyl)-2-nitrothiophene</td>
<td>C11H6Cl2N2O2S</td>
<td>301,15</td>
<td>+/-</td>
<td>cell death</td>
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</tr>
<tr>
<td>5-([1E]-2-[4-iodophenyl]-2-azavinyl)-2-nitrothiophene</td>
<td>C11H7N2O2S5</td>
<td>358,16</td>
<td>cell death</td>
<td>cell death</td>
<td></td>
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<tr>
<td>6-(tert-butyl)-2-[3-(tert-butyl)-5-bromo-2-hydroxyphosphinyl]-4-bromophenol</td>
<td>C20H24BrO2S</td>
<td>488,28</td>
<td>cell death</td>
<td>cell death</td>
<td></td>
</tr>
<tr>
<td>[5-nitro-2-thienyl]methylene)methane-1,1-dicarbonitrile</td>
<td>CBH3N3O2S</td>
<td>205,2</td>
<td>cell death</td>
<td>cell death</td>
<td></td>
</tr>
<tr>
<td>9-([1E]-2-nitrovinyl)anthracene</td>
<td>C16H11N02</td>
<td>249,27</td>
<td>cell death</td>
<td>cell death</td>
<td></td>
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<tr>
<td>di2,3,4,5,6-pentafluorophenyl ketone</td>
<td>C13F10O</td>
<td>362,13</td>
<td>cell death</td>
<td>cell death</td>
<td></td>
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Chemical compounds that repetitively induced cell death (upper panel) or functioned as radiosensitizers (lower panel) are represented. NPC=neural precursor cells; OK= no cell death as compared to control, +/- = ≤ 50% cell death.
Figure 2. A small molecule screen identifies quercetin as a radiosensitizer in medulloblastoma cells. (A) Example of a scatter plot of Gluc values (left panels) and Acumen read-out (right panels), representing cell survival after treatment of DAOY cells with 1 µM of the ActiTarg-K960 small molecule library in the presence, or absence of irradiation (4 Gy). The fluorescence in the single wells as measured by the Acumen is represented as relative intensities of the color green, where black corresponds with little cells and green with many cells. When using Gluc as a read-out, cell viability was measured by luciferase activity and corrected for the toxicity of the solvent, 0.1% DMSO (set to 100%). A reduction of >75% of cell growth was considered to be significant, as indicated by a dashed line. Each dot represents a single well; in position F05 a cytotoxic agent is identified, in position C09 a radiosensitizer. A representative 96-well plate is shown. (B) Structure formulae of compounds that induce cell death in DAOY medulloblastoma cells but show limited cytotoxicity on primary human fibroblasts and on C17.2 neuronal precursor cells (NPCs). Two radiosensitizing agents and three cytotoxic agents are shown. (C) A representative 96-well plate is shown. Each dot represents a single well; in position F05 a cytotoxic agent is identified, in position C09 a radiosensitizer. A representative 96-well plate is shown.
medulloblastoma. Therefore, we investigated if similar effects could be observed in additional medulloblastoma cell lines. D283-med, D458-med, and DAOY cells were incubated with 1 µM quercetin 30 minutes prior to irradiation, and cell numbers were determined at 4 days after treatment (Fig. 3A). Although the responses were less pronounced in the D458-med cells, a radiosensitizing effect of quercetin was observed in all cell lines, while quercetin treatment by itself did not inhibit cell proliferation. To further confirm the radiosensitizing potential of quercetin, dose-dependent clonogenic survival assays were performed. DAOY cells appeared unfit for these experiments, since they did not form clones. However, treatment of D283-med and D458-med medulloblastoma cells with 0.5 µM or 1 µM quercetin showed radiosensitization in both cell lines (Fig. 3B), even at radiobiologically relevant irradiation doses of 1 Gy or 2 Gy used in fractionated radiation schemes. These results strengthen our hypothesis that quercetin is a putative radiosensitizer for medulloblastoma. Next, we tested the radiosensitizing effect of quercetin on two primary medulloblastoma cell cultures, VU371 and ICb-1299MB. Similar as for the cell lines, cells were treated with 1 µM quercetin 30 minutes prior to irradiation, and viability was determined four days after treatment (Fig. 3C). Again, quercetin by itself did not reduce cell viability, however, these cells are highly sensitive to radiation (data not shown). Therefore, cells were irradiated with the lowest technical dose possible, 0.7 Gy, which already significantly impaired viability. We did not observe an enhanced radiation response in combination with quercetin in these cells. Since medulloblastomas have been subclassified into four molecular subgroups, we determined to which subgroups the used cell lines belong. Expression of four subgroup classifier genes was assessed by qRT-PCR, which was previously described by Zhao et al. (Supplementary Fig. S2A+B). DAOY was identified as a SHH medulloblastoma while D283-med, D458-med, and VU371 seem to belong to the group 3 subtype (Fig. 3D and Supplementary Fig. S2A+B). ICb-1299MB was previously classified as a group 4 medulloblastoma.

Quercetin treatment improves radiation efficacy in a xenograft tumor model

To further evaluate if quercetin can function as a novel agent in the treatment of medulloblastoma, we investigated the effect of this compound in an in vivo setting, using an orthotopic xenograft mouse model. Therefore, luciferase-expressing D283-med cells were implanted stereotactically into the left cerebellar hemisphere of nude mice. In two independent experiments, 31 out of 36 mice developed primary tumors. (C) Graphic representation of relative cell survival in DAOY medulloblastoma cells (left panel), primary human fibroblasts (PHF, middle panel), or neural precursor cells (NPC, right panel) after quercetin (QC) treatment and/or irradiation (IR), as extracted from the small molecule screen. Data are presented as means ± SD (n=3). ** p<0.005, Mann-Whitney U test.
Figure 3. Quercetin sensitizes towards irradiation in a panel of medulloblastoma cells. (A) Graphic representation of relative cell survival of DAOY, D283-med, and D458-med cells after 4 days of quercetin treatment in the presence or absence of irradiation (4 Gy). Cell numbers were determined by visual counts, using a Bürker hemacytometer. Vehicle treated cells are set at 100%. Data are presented as means ± SD (n=3). ** p<0.005, Mann-Whitney U test. (B) Clonogenic survival of D283-med (left panel) and D458-med (right panel) medulloblastoma cells, 14 days after irradiation (0-3 Gy). Cells were treated with 0 µM (●), 0.5 µM (○), or 1 µM (▼) quercetin 30 minutes prior to irradiation. A representative experiment is shown. (C) Graphic representation of relative cell survival of VU371 and Icb-1299MB cells after 4 days of quercetin treatment in the presence or absence of irradiation (0.7 Gy). Cell viability was determined by Cell Titer Glo assay. Vehicle treated cells are set at 100%. Data are presented as means ± SD. (D) Molecular subgroup classification of DAOY, D283-med, D458-med, VU371, and Icb-1299MB cells. Expression levels of subgroup classifiers WIF1, SFRP1, NPR3, and KCNA were determined by qRT-PCR to determine to which subgroups the used medulloblastoma cells belong.

These mice were then randomly assigned into four groups, and treated with vehicle (5% DMSO) or quercetin, either alone or in combination with irradiation. Since quercetin has a half-life of only 20 minutes in vivo\textsuperscript{29}, six consecutive injections with quercetin (100 mg/kg) were given at different time points around the time of irradiation (60 or 30 minutes before, 30 or 60 minutes after irradiation, 0 hours and 24 hours after irradiation). Quercetin treatment did not induce any neurological symptoms or other adverse events. Tumor growth was monitored twice a week by BLI. In addition, the mice were monitored daily for discomfort and weight loss. Although the BLI signal of the tumors did not differ between the various groups, survival analysis indicated a significant extension of the group that received ionizing radiation in combination...
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Figure 4. Effect of quercetin treatment in combination with irradiation on survival in a xenograft mouse model. (A) Kaplan-Meier survival analysis of medulloblastoma-bearing mice treated with quercetin (blue line), vehicle (5% DMSO – black line), vehicle and irradiation (green line), and quercetin in combination with irradiation (red line). A significant survival extension of the group that received ionizing radiation in combination with quercetin as compared to the vehicle-treated group (p=0.0052), the quercetin group (p<0.0001), or the group that only received radiotherapy (p=0.002; Fig. 4A). All animals treated with the combination of quercetin and irradiation survived for more than 24 days after treatment, with a median survival time of 32 days. Animals that did not receive irradiation (either vehicle- or quercetin-treated) had a median survival of only 12-17 days (Fig. 4B).

DISCUSSION

In the past decade, a better understanding of the biology and heterogeneity of childhood medulloblastomas has allowed an improved patient stratification and risk-adapted treatment strategies. However, despite these novel insights, therapy still fails in approximately 30% of patients and is often accompanied by severe long-term sequelae. Thus, there still is a need for alternative therapies that allow to lower the total dose of irradiation, reducing the long-term side effects, and/or increase the radiation efficacy. We show here that the flavonoid quercetin can sensitize medulloblastoma cells to irradiation, and that administration of quercetin during radiotherapy significantly improves survival in mice harboring medulloblastoma.

Quercetin was identified as a ‘ready-to-use’ radiosensitizer in a small molecule screen for medulloblastoma cells, using two independent read-out systems. The small molecule screen consisted of 960 compounds with a diversity of chemical structures that have been reported to exert kinase inhibitory properties. In this screen 23...
compounds were repeatedly identified that could inhibit cell growth (n=12), or sensitize towards irradiation (n=11). Strikingly, two of the compounds that induced cell death independently of irradiation had similar structures, as did three of the 11 compounds that functioned as a radiosensitizer (nitrophenes, Supplementary Fig. S1). However, most of those drugs also induced cell death in primary human fibroblasts or neuronal progenitor cells, rendering them unfit for the development of novel and tumor-specific therapies. Unlike those compounds, quercetin did not affect the proliferation of neuronal precursor cells or normal human fibroblasts, nor showed any toxicity in the absence of ionizing radiation. This is in concordance with previous studies that report selective activity of quercetin as a sensitizer to chemotherapeutics on cancer cells, but not in normal cells, even though higher quercetin concentrations (ranging from 5-200 µM) were used in these studies. Importantly, the low micromolar concentrations used in our experiments are in the range of the plasma concentrations that can be reached in humans and are considered to be safe. Quercetin is found in a broad range of fruits and vegetables such as apples, onions and tomatoes, and present in plasma at the nanomolar range (<100 nM) through our dietary intake, but micromolar concentrations have been reported after supplementation.

The observation that the concentrations needed for therapeutic benefit can be easily achieved by oral administration, and the fact that quercetin is cheap and readily available, renders this flavonoid an interesting option for the treatment of children with medulloblastoma. Another motive to consider quercetin as a radiosensitizer for this type of brain tumors, is the previously reported observation that quercetin can pass the blood-brain barrier (BBB). The BBB is a natural boundary between circulating blood and cerebrospinal fluid that protects the brain from toxins and potentially harmful substances (reviewed by Agarwal et al.). Although beneficial under normal circumstances, the presence of this BBB constitutes a major obstacle for drug delivery in the treatment of brain tumors. Importantly, quercetin has not only been shown to pass the BBB in in vitro systems, but has also been shown to accumulate in the brain after oral administration in rats. Moreover, quercetin has been shown to function as a neuroprotective agent, both in vitro and in vivo following ischemia, trauma, or other forms of induced brain damage. Of particular interest in the context of medulloblastoma treatment is the observation that administration of quercetin can improve learning and memory deficits in animals that were subjected to brain damaging agents.

The neuroprotective effect of quercetin has been suggested to be due to its anti-oxidative properties. Quercetin is a potent anti-oxidant that can scavenge free radicals and bind transition metal ions. However, alternative mechanisms, such as modulation of signal transduction pathways or effects on gene expression have also been reported. Quercetin can modulate the activity of many kinases and other enzymes (reviewed by Russo et al.), which may explain the diversity of its actions. Besides neuroprotective properties, quercetin has been described to prevent cardiovascular...
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diseases, to function as a chemopreventive agent, to have anti-proliferative and
growth-suppressing effects, to induce senescence and autophagy, and to have
anti-inflammatory and anti-angiogenic activities. Each property or action appears to
depend on the dose and model system used. Of note are the inhibition of the Wnt/β-
catenin pathway, which plays an important role in one of the four medulloblastoma
subgroups, and the inhibition of the Hepatocyte Growth Factor (HGF)-induced
cell migration in medulloblastoma by quercetin. Furthermore, quercetin has been
reported to induce radiosensitization in tumor cells other than medulloblastoma by
targeting the ATM-mediated pathway, which is critical in the DNA damage response.
Which pathway or mechanism is involved in the radiosensitization of medulloblastoma
cells by quercetin is not exactly known at the moment.

Four distinct molecular subtypes of medulloblastoma have been identified. Subclassification of the cell lines used in this study showed that the SHH and group 3 subtypes were represented. DAOY was identified as a SHH medulloblastoma, which was also suggested by Pambid et al. D283-med and D458-med belong to the group 3 medulloblastomas as was reported before for D458-med. We observed radiosensitization after treatment with quercetin in DAOY and D283-med and, to a lesser extent, in D458-med cells. This indicates that the mechanism underlying the improved radiation response caused by quercetin may represent a general effect on medulloblastoma cells and, thus, is likely to be subtype-independent. The primary cell cultures used in this study belong to the group 3 and group 4 subtypes. We did not detect a radiosensitizing effect of quercetin in these cells, since these cells are highly radiosensitive in vitro and there is no window in which quercetin can exert its effect. This was also seen in D458-med cells, which show high sensitivity to radiation. It would be of interest to examine the efficacy of quercetin in radioresistant primary medulloblastoma cell cultures. In addition, the response of primary cell cultures in vitro may not necessarily represent the response in the cerebellar environment. Therefore, we are setting up more primary cell lines of the different subtypes and use these to develop orthotopic mouse models to study the effect of quercetin as a radiosensitizer in medulloblastoma in an environment that is more comparable to that of the original tumor. However, the results presented here indicate that quercetin functions as a potent radiosensitizer in medulloblastoma cells at easily achievable concentrations, providing a promising lead for treatment of children with this type of brain tumor.

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**SUPPLEMENTARY INFORMATION**

Supplementary Figure S1. Structure formulae of compounds that induce cell death in DAOY medulloblastoma cells, as identified by a small molecule screen. Chemical compounds that repetitively induced cell death (upper twelve) or functioned as radiosensitizers (lower structures) are represented.
Supplementary Figure S2. Expression levels of subgroup classifiers WIF1, SFRP1, NPR3, and KCNA in medulloblastoma cells compared to normal cerebellum. (A) Expression levels of WIF1, SFRP1, NPR3, and KCNA in DAOY, D283-med, and D458-med compared to normal cerebellum. DAOY was identified as a SHH medulloblastoma, D283-med and D458-med were identified as group 3 medulloblastomas. (B) Expression levels of WIF1, SFRP1, NPR3, and KCNA in VU371 cells from a xenograft tumor and VU371 cells from the original tumor as compared to expression in normal cerebellum. VU371 was identified as a group 3 medulloblastoma. The subgroup type was preserved after serial transplantation of tumor cells in the cerebellum of mice.