Research paper

Celastrol-induced degradation of FANCD2 sensitizes pediatric high-grade gliomas to the DNA-crosslinking agent carboplatin

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\textbf{Abstract}

\textbf{Background:} Pediatric high-grade gliomas (pHGG) are the leading cause of cancer-related death during childhood. Due to their diffuse growth characteristics, chemoresistance and location behind the blood-brain barrier (BBB), the prognosis of pHGG has barely improved in the past decades. As such, there is a dire need for new therapies that circumvent those difficulties. Since aberrant expression of DNA damage-response associated Fanconi anemia proteins play a central role in the onset and therapy resistance of many cancers, we here investigated if FANCD2 depletion could sensitize pHGG to additional DNA damage.

\textbf{Methods:} We determined the capacity of celastrol, a BBB-penetrable compound that degrades FANCD2, to sensitize glioma cells to the archetypical DNA-crosslinking agent carboplatin \textit{in vitro} in seven patient-derived pHGG models. In addition, we tested this drug combination \textit{in vivo} in a patient-derived orthotopic pHGG xenograft model. Underlying mechanisms to drug response were investigated using mRNA expression profiling, western blotting, immunofluorescence, FANCD2 knockdown and DNA fiber assays.

\textbf{Findings:} FANCD2 is overexpressed in HGGs and depletion of FANCD2 by celastrol synergises with carboplatin to induce cytotoxicity. Combination therapy prolongs survival of pHGG-bearing mice over monotherapy and control groups (P<0.05). In addition, our results suggest that celastrol treatment stalls ongoing replication forks, causing sensitivity to DNA-crosslinking in FANCD2-dependent glioma cells.

\textbf{Interpretation:} Our results show that depletion of FANCD2 acts as a chemo-sensitizing strategy in pHGG. Combination therapy using celastrol and carboplatin might serve as a clinically relevant strategy for the treatment of pHGG.

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\textbf{Research in context}

\textbf{Evidence before this study}
Pediatric high-grade gliomas (HGG) are highly malignant brain tumors with a devastating prognosis, contributing to the most cancer-related mortalities in children. Expression of FANCD2, a member of the Fanconi anemia group genes, known to be involved in DNA repair, has been linked to glioma grade in adults. In addition, inhibition of FANCD2 has been shown to induce cisplatin sensitivity in lung cancer models \textit{in vitro}. Celastrol is a natural compound that has been shown to degrade FANCD2 via activation of the proteasome. Several studies propose celastrol as a suitable compound against Alzheimer’s disease progression, by highlighting its neuroprotective properties and ability to cross the blood-brain barrier, which contributes to the translational potential of celastrol in brain tumor studies.

\textbf{Added value of this study}
Our study identifies FANCD2 as a therapeutic target in pediatric HGG. We developed a synergistic treatment strategy that...
uses the brain bioavailable FANCD2 inhibitor celastrol to sensitize pHGG to the classical chemotherapeutic carboplatin. A genetically diverse panel of primary patient-derived pHGG cultures all showed a synergistic antitumor response to this drug combination. Moreover, survival of mice carrying primary patient-derived pHGG xenografts was significantly prolonged by combined treatment with celastrol and carboplatin. Altogether, these findings may lead to the development of a multimodal therapeutic strategy for pHGG based on FANCD2 inhibition.

Implications of all the available evidence

This study shows an effective treatment in pediatric high-grade gliomas in vitro and in vivo. However, as FANCD2 overexpression is also detected in adult high-grade gliomas, this treatment strategy might be extrapolated to this patient group as well. However, since pure celastrol may have a narrow therapeutic window, ideally alternative FANCD2 inhibitors or synthetic celastrol analogues should be used for clinical translation. Furthermore, future studies should investigate if other classes of DNA damaging agents can be more effective in co-treatment with celastrol.

1. Introduction

Pediatric high-grade gliomas (pHGG) are among the most lethal malignancies in children. Despite recent advances in understanding the molecular basis of these tumors, clinical prognosis for pHGG patients has not yet improved. pHGG are characterised by epigenetic alterations and defects in DNA damage repair genes. However, little is known about non-mutated DNA-damage repair genes that are deregulated in pHGG.

Disruption of the Fanconi DNA-repair mechanism has been linked to the onset of several malignancies through a syndrome called Fanconi anemia (FA), a genetic disorder caused by germline mutations in any of the genes coding for one of the Fanconi proteins [1–3]. FA is associated with genomic instability and strong sensitivity to DNA-crosslinking analogues, because of the absence of an operational Fanconi machinery [4]. Fanconi anemia group D2 (FANCD2), one of the core components of the Fanconi repair mechanism, induces an intracellular response to DNA damage upon complexing with FANCI [5]. In contrast to cancers harbouring mutated Fanconi components, little is known about cancers with deregulated, yet non-mutated, Fanconi core components.

In this study we show that FANCD2 expression levels increase with glioma grade, as is supported by earlier findings [6]. We hypothesised that HGGs are highly dependent on the Fanconi system for DNA repair and that disruption of this mechanism would make these tumors prone to DNA-crosslinking agents, as seen in FA patients [4].

To study if depletion of the deregulated FANCD2 in pHGG cells could cause a FA-like phenotype that leads to a higher sensitivity to platina analogues, we treated primary pHGG cells with carboplatin in combination with celastrol, a blood-brain barrier (BBB) permeable compound that has been shown to cause degradation of FANCD2 via the proteasome [7,8]. In our experiments, carboplatin was chosen over other platinum chemotherapeutics because of its relatively mild neurotoxic side effects and reported ability to cross the BBB [9,10].

Here we show that depletion of FANCD2 by celastrol treatment provokes stalled fork replication and causes sensitivity to the DNA-crosslinking agent carboplatin in primary pHGG cells. Furthermore we show that treatment of mice carrying primary patient-derived pHGG xenografts with this combination of drugs leads to prolonged survival, as compared to monotherapy.

2. Materials and methods

2.1. Cell cultures

HSDJ-DIPG-07 [11] (H3.K27M, DIPG) was a kind gift from Dr Montero Carcaboso (Hospital San Joan de Déu, Barcelona, Spain), JHH-DIPG-01 [12] (H3.K27M, DIPG) from Dr Raabe (John Hopkins Hospital, Baltimore, USA), and SU-DIPG-IV (H3.1K27M, DIPG) and SU-pcGBM2 (H3 wildtype, GBM) [13] from Dr Monje (Stanford University, Stanford, USA). VUMC-DIPG-10 [14] were cells established at the VU University Medical Center from a DIPG autopsy (H3 wildtype), while VUMC-DIPG-F [15] and VUMC-DIPG-G are biopsy-derived H3.K27M DIPG cultures. VUMC-HGG-05 [16] (giant-cell GBM), VUMC-HGG-09 (IDH1 R132H) and VUMC-HGG-14 are resection-derived glioblastomas. VUMC-HGG-11 [17] is a thalamic midline glioma harbouring a H3.3 K27M mutation. All models are FANCD2 wild-type as confirmed by whole-genome sequencing.

Cells were cultured as neurospheres as previously described [15,17,18]. Cells were only used when confirmed mycoplasma negative and short-tandem repeat (STR) analysis was performed to ensure cell line identities.

2.2. Human mRNA expression datasets

The following expression datasets were used to study mRNA expression between patients:

Healthy brain (GSE 11882 and 13564) [19,20], healthy cerebellum (GSE 3526) [21], glioma (GSE 16011 and 4290) [22,23], glioblastoma (GSE 7696) [24], pediatric HGG (GSE 19578) [25], pediatric DIPG (GSE 26576) [26]. Survival correlation to FANCD2 expression was studied using the glioma dataset GSE 43378 [27].

2.3. Chemicals

Celastrol (CAS 34157-83-0), carboplatin (CAS 41575-94-4), MG-132 (CAS 133407-82-6), and topotecan (CAS 123948-87-8) were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). For in vitro studies, celastrol, topotecan and MG-132 were dissolved in DMSO and stored at a 10 mM concentration. Carboplatin was stored as a 10 mM stock dissolved in H2O for increased stability compared to storage in DMSO [28]. For in vivo studies, celastrol was dissolved in DMSO, and carboplatin was dissolved in 0.9% saline.

2.4. Cell viability assays

For cell viability assays, cells were plated at a density of 5000 cells/well in 96-well F-bottom cell-repellent plates (Greiner Bio-one, #650971). 24 h after cell seeding, drugs were dispensed at different concentrations using a Tecan D300e picoliter dispenser (Tecan Group Ltd, Switzerland) and incubated at 37 °C and 5% CO2 for 96 h. CellTiter-Glo® 3D Luminescent Cell Viability Assay (Promega) was used as a method to determine the number of viable cells in culture following manufacturer’s protocol. Luminescence was measured using a Tecan Infinite® 200 reader using iControl 1.10 software. Synergy scores are based on average cell viability at 96 h after treatment at the indicated concentrations and were calculated using the Synergy finder software.

2.5. Western blotting

Immunoblotting was performed as previously described [15]. Protein was isolated using RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Membranes were incubated with Rabbit anti-FANCD2 (1:1000, Abcam, Cambridge, UK, #ab108928), mouse anti-phospho-Histone H2AX (Ser139) (1:2000, Millipore, Burlington, MA, USA, #05-636), rabbit anti-RAD51 (D4B10) (1:1000, Cell
2.6. RNA sequencing

JHH-DIPG-01, HSJD-DIPG-07, SU-pcGBM2, and SU-DIPG-IV neurospheres were treated with 100 nM celastrol, 1 μM carboplatin, or a combination thereof. After 24 h, cells were collected and RNA was extracted using the miRvana miRNA isolation kit without phenol (Ambion, Life Technologies, NL), supplemented with Acid-Phenol: Chloroform. Subsequently, RNA-quality was analyzed with the Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit (Thermo Fisher, Waltham). Only samples that received a RIN>7 were further processed.

Sequencing was performed using an Illumina Nextseq 500 sequencer according to the manufacturer’s instructions. Alignment, feature counts, and differential expression were analyzed using the RNA-Seq v5 pipeline (GenomeScan, Leiden, NL). The human Ensembl GRCh37.75 reference was used for alignment of the reads for each sample. The reads were mapped to the reference sequence using a short read aligner based on Burrows-Wheeler Transform (TopHat v2.0.14) with default settings. The read counts were loaded into the DESeq2 package v1.14.1 within the R platform v3.3.0 for statistical analysis. Fastq files were uploaded to the R2 platform (r2.amc.nl) for further analysis and statistics. A list of all differentially expressed genes between the non-treated and celastrol treated groups is added as supplementary file 1.

2.7. Immunofluorescent imaging

VUMC-HGG-09 cells were cultured in TSM supplemented with FCS in Greiner SCREENSTAR® 96-well plates (#655-866) specialised for fluorescent imaging. Cells were treated with celastrol, carboplatin or a combination thereof for 12 h. Hereafter, cells were washed with ice cold PBS and fixed in 4% paraformaldehyde. After blocking (PBS +1% BSA for 30 min) and permeabilisation (PBS +0.1% Triton-X for 30 min), primary antibodies, rabbit anti-FANCD2 (1:1000; Abcam, #ab108928), and mouse Anti-Phospho-Histone H2AX (Ser139) (Clone SJ4a, Millipore, #05-636), were added to the slides for overnight incubation at 4°C. As secondary step Alexa Fluor® 488 (goat anti-rabbit, 1:10,000, Invitrogen, #411-667) and Alexa Fluor® 594 (goat anti-mouse, 1:10,000, Invitrogen, #1887003) were incubated for 1 h at RT. Imaging was done using the Zeiss Axioobserver Z1 inverted microscope using a 40x and 63x objective equipped with a Hamamatsu ORCA AG Black and White CCD camera. Quantification of yH2AX foci was done using ImageJ software, according to the automatic particle analysis for cell counting (200μp²), followed by automatic single point selection overlay (noise tolerance 45).

2.8. Neutral comet assay

To detect DNA double-strand breaks (DSBs), neutral comet assays were performed as previously described [29]. Shortly, cells were harvested, 8000 cells were diluted in 400 μl PBS and embedded in 1.2 ml 1% low-gelling agarose (Sigma). 100 μl of the cell suspension was used to make gels onto Trevigen comet assay slides. To lyse the cells in the gel, slides were incubated in lysis solution (2% sarkosyl, 0.5 M Na2EDTA and 0.5 mg/ml Proteinase K) overnight at 37°C. The next day, slides were rinsed three times for 30 min at room temperature in electrophoresis buffer (90 mM TrisHcl pH = 8.5, 90 mM Boric Acid and 2 mM Na2EDTA). Electrophoresis was performed for 25 min at 20 V in electrophoresis buffer. Subsequently, slides were washed once with distilled water. To stain DNA slides were incubated with 2.5 μg/ml Propidium iodide diluted in distilled water for 20 min. Individual comets were imaged with a Zeiss AxioObserver Z1 inverted microscope using a 20x objective equipped with a Hamamatsu ORCA AG Black and White CCD camera. Tailmoments of individual comets were assessed using the CASP software (http://casplab.com/). For each condition, more than 50 cells were analyzed.

2.9. DNA fiber assay

DNA fiber assays were performed as previously described [30]. Briefly, cells were pulse labelled with 25 μM CldU followed by 250 μM IdU for 30 min each. Labelled cells were trypsinised, lysed in spreading buffer (200 mM Tris-Hcl pH7.4, 50 mM EDTA and 0.5% SDS) and spread on microscope slides (Menzel-Gläser, Superfrost). DNA fibers were fixed on slides using 3:1 methanol: acetic acid. Slides were treated with 2.5 M HCl for 1 h and 15 min to denature DNA followed by 1 h incubation in blocking buffer (PBS, 1% BSA, 0.1% Tween20). For detection of CldU and IdU, slides were incubated for 1 h with rat-anti-Brdu (Clone BU1/75, Abcam; 1:500) and mouse-anti-BrdU (clone B44, Becton Dickinson; 1:750), respectively. Subsequently, slides were fixed with 4% paraformaldehyde for 10 min and incubated with Alexa 488-labeled goat-anti-mouse and Alexa 555-labeled goat-anti-rat (Molecular probes; 1:500) for 1.5 h. DNA fibers were imaged with the Zeiss AxioObserver Z1 inverted microscope using a 63x objective equipped with a Hamamatsu ORCA AG Black and White CCD camera. Replication track lengths were analyzed using ImageJ software and the conversion factor 1 μm = 2.59 kb was used [31].

2.10. Establishment of stable FANCD2 knockdown cells

FANCD2 knockdown cells were established using the pLKO.1-shFANCD2.1 and pLKO.1-shFANCD2.2 (GE Healthcare, Chicago, USA) plasmids as described previously [17]. shRNA sequences are summarised in supplementary table S1.

2.11. In vivo experiments

4-week old female athymic nude mice (FVB foxn1−−−−) were purchased from Charles River Laboratories (‘s-Hertogenbosch, NL) and housed in compliance with European and national guidelines for experimental animal research (protocol #841-NCH17-04, animal welfare committee, Vrije Universiteit, Amsterdam). After one week of acclimatization, VUMC-HGG-14 cells (50×10⁶ cells in 5μL) were stereotactically injected into the striatum (bregma:x-2 mm;y:0.5 mm;z:3 mm), and tumor growth was followed longitudinally by bioluminescence imaging (BLI) using the IVIS Spectrum (Perkin Elmer, Waltham, MA, USA). When tumor growth showed a stably increasing signal in all animals, mice were stratified into four groups based on absolute BLI signal and signal increase between last two measurements. Group A received saline (0.9%) intravenously (i.V.) and water via oral gavage (O.G.). Group B received carboplatin (150 mg/kg) once a week via i.V. injection [32] and water orally. Group C received celastrol (2.5 mg/kg) via O.G. [33] daily for five consecutive days, and after one week off-treatment a second five day treatment round, this time of 5 mg/kg celastrol per day. Group D received 0.9% saline via i.V. injection. Group D was treated with both celastrol and carboplatin as described for group B and C. Survival was scored based on humane endpoints, which were defined as a >20% loss of body mass from highest weight or severe neurological deficits. Only mice that experienced tumor metastasis into the ventricles within two weeks after injection were excluded from the study, as these were considered implantation errors.
Table 1
Overview of the pediatric high-grade glioma models used in this study.

| Model            | Age | Location | Diagnosis | H3 mutation   | Other mut.            | Treatment                                      |
|------------------|-----|----------|-----------|---------------|-----------------------|------------------------------------------------|
| VUMC-DIPG-F      | 7y  | Pons     | DIPG      | H3.K27M       |                       | Biopsy (no treatment)                           |
| VUMC-DIPG-G      | 11y | Pons     | DIPG      | H3.K27M       | TP53                  | Biopsy (no treatment)                           |
| VUMC-DIPG-05     | 11y | Cortical | GBM       | WT            |                       | Resection (no treatment)                        |
| VUMC-DIPG-10     | 12y | Pons     | DIPG      | WT            |                       | N1-1                                           |
| VUMC-HGG-09      | 11y | Cortical | GBM       | WT            |                       | Biopsy/resection (no treatment)                 |
| VUMC-DIPG-10     | 12y | Pons     | DIPG      | WT            |                       | XRT+gemcitabine                                |
| VUMC-HGG-11     | *   | Thalamus | Thalamic glioma | H3.K27M | TP53                  | Biopsy/resection (no treatment)                 |
| VUMC-HGG-14     | 17y | Cortical | GBM       | WT            |                       | BRAF, Biopsy/resection (no treatment)           |
| HSJD-DIPG-07     | 6y  | Pons     | DIPG      | H3.K27M       | ACVR1                 | Autopsy (no treatment)                          |
| JHH-DIPG-01     | 8y  | Pons     | DIPG      | H3.K27M       |                       | Autopsy, XRT+carboplatin/etoposide, sorafenib, erlotinib, irinotecan/temozolomide, irinotecan/bevacizumab |
| SU-DIPG-IV       | 6y  | Pons     | DIPG      | H1.K27M       | ACVR1, TP53           | Autopsy, XRT+taxotere/irinotecan                |
| SU-pcGBM2        | 15y | Cortical | GBM       | WT            |                       | Diagnosis sample (no treatment)                 |

* = Age unknown.

Upon reaching their humane endpoint, mice were euthanised by Euthasol® 20% (AST Farma, Oudewater, NL), after which brains were collected and fixed in 4% paraformaldehyde for 48 h. The fixed brains were embedded in paraffin and processed for human-vimentin immunohistochemical staining (mouse-anti-human-vimentin, clone V9, #M0725, DAKO, Santa Clara, USA). Human-vimentin stainings were imaged using the Vectra® PolarisTM Automated Quantitative Pathology Imaging System (Perkin Elmer, Waltham, USA). Furthermore, the brains were processed for KI-67 and cleaved caspase-3 immunohistochemical stainings (rabbit-anti-KI-67, clone D2H10, and rabbit-anti-cleaved caspase 3, clone ASP175, #9661S, both from Cell Signaling Technology). KI-67 and cleaved caspase-3 stainings were imaged using a Zeiss Axio optical microscope equipped with a Zeiss Axiocam ICC5 operated by ZEN Pro-imaging software. All brains used for these stainings originate from mice that were euthanised approximately three weeks after finishing the full treatment protocol.

3. Results

3.1. FANCD2 is overexpressed in pediatric high-grade gliomas

To evaluate if expression of Fanconi complex genes correlates with glioma-grade, we analyzed mRNA expression of Fanconi genes in publicly available gene expression datasets. Therefore, we used the R2 platform (r2.amc.nl), a database that includes gene expression profiles from large patient cohorts, including tools to compare gene expression between different cancer types. We found that the expression of FANCD2 in HGG tumor tissues is upregulated compared to healthy brain tissues (172 healthy brain tissues and 9 healthy cerebellum tissues compared to 368 adult HGG and 90 pediatric HGG tumor samples), as depicted in Fig. 1a. On average, tumor tissue showed a 12-fold upregulation of FANCD2 compared to healthy brain (P < 0.0001 for all glioma samples vs. healthy brain and cerebellum). Also, FANCD2 mRNA expression levels significantly correlated with glioma grade (Fig. 1b), as did patient survival (Fig. 1c). Furthermore, we found markedly less overexpression of other genes of the Fanconi complex in HGGs compared to healthy brain tissue (Supplementary figure S1), suggesting that in HGGs FANCD2 plays a unique role independently of the classical Fanconi complex mechanism.

To validate the patient cohort finding, we assessed FANCD2 protein expression profiles in a panel of pHGG cells by western blotting (Fig. 1d), using FANCD2 protein expression in human astrocytes as a healthy control. All pHGG models showed expression of FANCD2, albeit with different expression levels, while human astrocytes showed no visible FANCD2 protein expression. An overview, including details on the molecular background and treatment history, of all the pHGG models used in this study is depicted in Table 1.

3.2. FANCD2 is degraded upon celastrol treatment in primary pHGG cells

To test the effect of FANCD2 downregulation in our pHGG cell cultures, we used the plant derivate celastrol, which has been reported to induce degradation of FANCD2 via proteasome activity [8]. Therefore, we treated three different pHGG cultures with celastrol for 24 h and observed a strong decrease in FANCD2 protein expression in all models (Fig. 2a). We then compared RNA-sequencing results of four treated pHGG models (HSJD-DIPG-07, JHH-DIPG-01, SU-DIPG-IV, SU-pcGBM2) to their non-treated controls through a parametric analysis of gene set enrichment (PAGE), using the KEGG database. This analysis showed a significant differential expression of the proteasome gene set between the non-treated and celastrol-treated groups (FDR-corrected, P<0.05) (Fig. 2b), suggesting that celastrol treatment alters proteasome activity in our pHGG cultures. Furthermore, comparison between the non-treated and celastrol-treated groups revealed no difference in FANCD2 mRNA expression (P=0.41), suggesting that celastrol alters FANCD2 expression in a post-transcriptional manner (Supplementary figure S2). These results were further confirmed by treating two pHGG cultures with the proteasome inhibitor ‘MG-132’ [36], followed by celastrol treatment, which rescued FANCD2 protein expression (Fig. 2c). Together, these results imply proteasomal degradation as a mechanism that degrades FANCD2 in pHGG cells after celastrol treatment.

3.3. Celastrol and carboplatin produce synergistic cytotoxicity in primary pHGG cells

Because FANCD2 overexpression in pHGG suggested a Fanconi repair pathway dependency, we hypothesised that celastrol-driven FANCD2 degradation could sensitize pHGG cells to DNA-crosslinking...
agents. Therefore, we investigated the effect of celastrol and carboplatin monotherapy and a combination of both compounds on a panel of primary pHGG cell cultures. Cells were treated for 96 h with both compounds at a range of concentrations to determine cytotoxic IC50. Celastrol IC50 concentrations ranged from 0.5 μM to 3 μM (Fig. 3a, c), while carboplatin IC50 concentrations ranged between 10 and 24 μM (Fig. 3b, c). Importantly, combination treatment caused synergistically increased cytotoxicity, especially when semi-toxic
(IC-50) celastrol concentrations were combined with nontoxic carboplatin concentrations (Fig. 3d, e). Similar experiments with topotecan, an effective DNA-damaging topoisomerase inhibitor often used to treat GBM, instead of carboplatin in three pHGG cell lines (VUMC-HGG-05, JHH-DIPG-01, and HSJD-DIPG-07) showed no synergistic effect on cell viability (Supplementary Figure S3) [37].

FANCD2 knockdown by shRNA in pHGG cultures resulted in a similar sensitisation to carboplatin cytotoxicity as celastrol treatment, confirming the FANCD2-dependence of the observed synergy (Fig. 4a-b). As expected, celastrol monotherapy sensitivity was not effected in all FANCD2 shRNA pHGG cultures, suggesting the need for a trigger, like carboplatin, to take advantage of the absent repair machinery (Supplementary figure S4).

### 3.4. Celastrol induces genomic instability by arresting the replication fork

To determine whether the synergistic effect of celastrol was related to the DNA-damaging effect of carboplatin, we examined the expression of γH2AX by immunofluorescence after treatment with either compound as monotherapy, or in combination [38]. While celastrol monotherapy depleted FANCD2 expression, no accumulation of the DNA-damage marker γH2AX was observed. Treatment of VUMC-HGG-09 cells with carboplatin, however, caused nuclear accumulation of γH2AX (Fig. 5a, b, c). The number of foci increased when carboplatin treatment was combined with celastrol, leading to a further accumulation of γH2AX foci within the nucleus. Similar results were obtained for different pHGG cultures by western blotting, although in some cases an increase in γH2AX expression was observed upon celastrol monotherapy as well (Fig. 5d).

To confirm that the γH2AX accretion after celastrol and carboplatin combination therapy was the result of accumulation of double strand breaks (DSBs), we made use of the neutral comet assay that determines the length of DNA tails as a measure for DSBs [29]. Unexpectedly, in both the JHH-DIPG-01 and HSJD-DIPG-07 a significant (P<0.0001) increase in tail length was observed after celastrol treatment, while carboplatin treatment did not induce an increased amount of DSBs (Fig. 5e, f). Combination therapy induced a similar average tail length as the celastrol treated group, suggesting that celastrol is solely responsible for DSB accumulation after 24 h of treatment, which is paradoxical with our γH2AX measurements. However, other mechanisms may account for the differential accumulation of γH2AX foci between celastrol monotherapy and combination treatment, such as stability of the replication fork. Recent literature suggests a direct connection between FANCD2 expression and replication fork stability via RAD51 and SETD1A [39,40]. Therefore, we assessed the expression of these proteins before and after celastrol treatment by western blot. As shown in Fig. 5g-h, both RAD51 and SETD1A protein expression decreased after 24 h of celastrol treatment. Like the results in the cell-viability assays, downregulation of RAD51 upon celastrol treatment appears to depend on the presence of FANCD2, as stable knockdown of FANCD2 decreases protein levels of RAD51 as well (Fig. 5e).

To further confirm that celastrol caused stalled replication forks via depletion of FANCD2, we used a DNA fiber assay to analyze the DNA synthesis rate. After treating pHGG cells for 24 h with celastrol we found a significant (P<0.0001) decrease in replication fork progression in all cultures compared to controls (Fig. 5i), indicating that celastrol arrests the replication fork.

### 3.5. Celastrol and carboplatin combination treatment prolongs survival in a pHGG xenograft model

To determine the therapeutic efficacy of celastrol and carboplatin in vivo, we tested these compounds in athymic nude mice carrying patient-derived pediatric glioblastoma xenografts (VUMC-HGG-14). This model was established from surgical resection material of a GBM from a pediatric patient and shows a typical GBM-like growth pattern in the mice (Fig. 6a). VUMC-HGG-14 cells were transduced in vitro with luciferase in order to monitor intracranial tumor-burden in vivo in time and were validated using STR analysis. Treatment started 15 days after tumor implantation upon demonstration of engraftment by bioluminescence imaging (BLI), at which moment mice were stratified into four equivalent groups (control, carboplatin (150 mg/kg), celastrol (2.5 and 5 mg/kg), and combination) based on BLI signal intensity [41]. Since no toxicity was observed after the first treatment round, we increased the
Fig. 3. Celastrol sensitizes pHGG cells to carboplatin in vitro

(a) Dose-response curves representing cell viability of primary pHGG models after celastrol treatment for 96 h.

(b) Dose-response curves representing cell viability of primary pHGG models after carboplatin treatment for 96 h.

(c) IC50 values (in μM) for each cell line to celastrol and carboplatin mono therapy.

(d) Bar graph visualizing relative cell viability of seven primary pHGG models at 96 h of treatment with either 3 μM carboplatin, 2 μM celastrol, or the combination thereof. The combination treatments show a synergistic effect on cell viability compared to the monotherapies. Error bars represent +/− SEM (n = 6). Cell viability is relative to DMSO-treated controls.

(e) 2D (left) and 3D (right) visualization of average synergy between celastrol and carboplatin at various concentrations in the seven pHGG cell models used in Fig. 3c. ZIP synergy scores >0 indicate synergism (in red). Maximum calculated synergy score = 48,35 (scores >10 are considered highly synergistic).
celastrol dose from 2.5 mg/kg to 5 mg/kg in the second round. No treat-
ment-related toxicities were observed and all mice developed GBM.
Using 20% weight loss or the development of severe neurological de-
ficits as humane endpoints, we observed a signifi-
cantly increased survival of the celastrol/carboplatin combination therapy group compared to the control or monotherapy groups (median survival increase of 43% versus control; \( P < 0.05 \) for combination vs any other group (log-rank test), Fig. 6b). In the combination treatment group we observed a trend to-
ward sles sn eur ologic d e-
ficits, as based on dropout causes per group (Supplementary Figure S5a). Furthermore, we observed normal weight distributions between groups and found no trend in longitudinal BLI sig-
als (Supplementary Figure S5b-c). tumor propagation after treatment
was investigated by immunostaining the proliferation marker Ki-67.
Expression of Ki-67 was decreased upon celastrol and carboplatin com-
bination therapy, while no difference was observed in Ki-67 expression
between tumors treated with monotherapy and controls (Fig. 6c). Fur-
thermore, immunostaining of cleaved caspase-3 as a marker of apopto-
sis showed no difference between the four groups in both malignant
and non-malignant brain areas (Supplementary Figure S6).

4. Discussion

In this study we found that FANCD2 serves as a guardian against
carboplatin induced toxicity in pHGG. We used the natural
compound celastrol to induce degradation of FANCD2 in pHGG cells
and show that this creates a therapeutic window for the platinum
analogue carboplatin to induce an accumulation of interstrand cross-
links in glioma cells, leading to cytotoxicity.

Except for some reports in melanoma [42] and glioblastoma [6]
not much is known about deregulation of non-mutated Fanconi pro-
teins in cancer. The Fanconi core component FANCD2 seems primar-
ily overexpressed in more aggressively growing gliomas, and protein
expression has been shown to correlate with glioma grade [6]. This
observation is in line with our gene expression analysis, where we
find strong FANCD2 upregulation in HGGs compared to healthy brain
tissues. Also protein expression analysis showed FANCD2 overex-
pression in all our FANCD2 wildtype pHGG cell models compared to
astrocytes in vitro, independent of their molecular background or
treatment history. This overexpression seems specific for FANCD2, as
we did not find other Fanconi complex genes overexpressed to the
same extent in glioma gene expression datasets compared to healthy
brain tissues. This may indicate that FANCD2 plays a vital role in
HGGs that is distinct from the traditional Fanconi repair machinery,
which relies on Fanconi core complex formation [43,44].

In our in vitro pHGG models we observed that celastrol affects gli-
oma cell viability at lower concentrations than described in literature
for other cancer cell types [45]. This could be explained by the rela-
tively quick proliferation speed and higher mutational burden of
**Fig. 5.** Celastrol treatment impairs DNA-damage repair and stalls replication forks.

(a) γH2AX immunofluorescent stainings in VUMC-HGG-09 cells after drug treatment (celastrol 1 μM, carboplatin 3 μM) (24 h). γH2AX is depicted in green and DAPI in blue.

(b) Quantification of γH2AX foci per nucleus in VUMC-HGG-09 cells after drug treatment (celastrol 1 μM, carboplatin 3 μM) (24 h). P-values between each group are <0.0001, except for control vs. carboplatin (P = 0.08981).

(c) Immunofluorescent stainings of VUMC-HGG-09 cells after drug treatment (24 h). (a): untreated; (b): celastrol 1 μM; (c): carboplatin 3 μM; (d): combination. FANCD2 staining is depicted in magenta, γH2AX in green, and DAPI in blue.
Fig. 6. Celastrol and carboplatin combination therapy prolongs survival in a patient-derived pediatric glioblastoma xenograft model in vivo.

(a) Immunohistochemical staining of human vimentin (brown) in the brain of a VUMC-HGG-14 xenograft-bearing mouse, revealing a diffuse growth pattern typical of GBM. One representative brain slide is shown.

(b) Survival analysis of VUMC-HGG-14 orthotopic xenograft-bearing mice treated with vehicle (red line, n = 8), celastrol (blue line, n = 7), carboplatin (green line, n = 7) or a combination of both (black line, n = 6). The combination treated group shows significant benefit over the other 3 groups. *: P < 0.05, **: P < 0.005 (log-rank test) (n = 28).

(c) Human Ki-67 immunohistochemical staining (in brown) in VUMC-HGG-14 tumor areas in the brains of each mouse group. Edges of diffuse and nondiffuse areas are shown.

(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
HGGs compared to lower-grade tumors [46], through which HGGs might rely on higher FANCD2 expression levels for their replication errors to be restored. Furthermore, we show that celastrol effectively downregulates FANCD2 protein expression in a post-transcriptional manner, without leading to a complete loss of FANCD2 protein. This likely explains why celastrol treatment is so well tolerated in vivo, as healthy cells remain viable under low FANCD2 expression levels [47].

In order to confirm that celastrol causes FANCD2-mediated fork degradation, we used RAD51 and SETD1A as markers for this process. RAD51 has a central role in DNA-repair by promoting homologous recombination repair and acts as a facilitator for the formation of reversed replication forks, where it protects nascent DNA strands from nucleosome digestion [48]. Recent literature describes a direct link between FANCD2 and RAD51, via modification of histone methylation [39]. FANCD2 recruits histone chaperones that modulate histone mobilization, allowing for SETD1A mediated mono-methylation to occur at the histone 3 lysine 4 (H3K4) site. H3K4 mono-methylation is vital for histone accessibility onto the regressed arms of reversed forks, which stabilises RAD51 filaments at the 3’ end of nascent DNA. Since RAD51 prevents digestion of this nascent DNA by stabilizing it, RAD51 acts as a guardian against uncontrolled DNA resection. It is therefore hypothesised that post-transcriptional depletion of FANCD2 causes loss of RAD51 protein expression via lost H3K4 mono-methylation, which is the result of lost SETD1A protein. We therefore consider the loss of SETD1A and RAD51 as markers for increased genomic instability. Correspondingly, using the DNA-fiber assay we were able to confirm that celastrol treatment induces stalled replication forks, which contributes to genomic instability. Even though DNA-crosslinking, in contrast to DSB formation, seems the essential mechanism to achieve pHGG-specific cell death after celastrol treatment, we used yH2AX as a marker for DNA-damage. yH2AX is a widely used marker for DNA-DSBs or G2/M arrest [38,49]. However, DSBs are also the long term result of non-repaired DNA-crosslinks. Where yH2AX expression peaks seconds after induction of direct DSBs via irradiation, yH2AX levels peak 12 to 24 h after the addition of DNA-crosslinking agents [50]. When intracellular repair machineries fail to repair these interstrand crosslinks, degradation of DNA occurs and DSBs arise [51]. Furthermore, next to being a marker for DNA DSBs, yH2AX has been shown to be a marker for stalled replication forks [52]. Therefore, the yH2AX foci increase between celastrol versus combination treatment, while no increase in DSB between these groups is observed, could be the result of inter-strand-crosslink accumulation as a cause of stalled replication forks. These findings were further confirmed when we found no synergism between celastrol and topotecan, which is, like carboplatin, another clinically relevant DNA-damaging agent but without any interstrand-crosslink abilities.

In our study we used celastrol treatment to induce FANCD2 degradation. Celastrol is a natural plant-derivative that crosses the BBB and was found to have several physiological properties, including strong anti-inflammatory and antioxidant activities [53,54]. As celastrol treatment did not cause any adverse effects in vivo and has been used safely in traditional eastern medicine and has been used in patients for decades and the step to using this compound in HGG patients is highly feasible [10,55].

In vivo experiments using a glioblastoma orthotopic xenograft model showed a significant survival benefit for mice treated with celastrol and carboplatin combination therapy over monotherapy and non-treated groups. This correlated with a reduced expression of the proliferation marker Ki-67 in the combination therapy group, while no changes of cleaved caspase-3 were observed in both healthy and malignant tissues. While this pledges for an absence of treatment-induced neurotoxicity, care needs to be taken, as the brains used for these stainings were collected from the first dropout mice of each group, three weeks after treatment. This is a limitation of the study and future studies should histologically examine brain tissues during the treatment procedure, in order to show the direct effect of treatment on Ki-67 and cleaved caspase-3. Interestingly, we observed less, and later, severe neurological symptoms in the group treated with both celastrol and carboplatin, albeit as a non-significant trend. BLI imaging of tumor growth was stopped after 41 days due to the diffuse growth characteristics of the tumors, which affected the observed BLI signal based on its migration pattern. However, the results from this first in vivo trial, combined with our in vitro data, could serve as the basis for more in depth studies into the efficacy of celastrol and carboplatin combination therapy in molecularly different HGG models in vivo. Especially a more thorough in vivo investigation in H3.1K27 and H3.3K27 mutated DIPG models would be a valuable addition to the findings presented here.

In conclusion, we demonstrate that FANCD2 plays a vital role in maintaining genomic integrity in pHGG cells and that degradation of FANCD2 via celastrol treatment sensitizes HGGs to carboplatin-mediated DNA damage. Our study demonstrates that FANCD2 knockdown in HGGs causes stalled replication forks which disables the tumor cells to unwind DNA-crosslinks. Since HGGs are highly proliferative and depend on fork-mediated DNA-repair mechanisms, we propose FANCD2 as a novel candidate for therapeutic intervention in HGGs.

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Declaration of Competing Interest

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Dennis S. Metselaar: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Visualization, Writing - original draft. Michael H. Meel: Conceptualization, Methodology, Writing - original draft. Bente Benedict: Investigation, Methodology, Writing - original draft. Piotr Waranneck: Investigation, Writing - original draft. Jan Koster: Formal analysis, Software, Writing - original draft. Gertjan J.L. Kaspers: Writing - original draft, Supervision. Esther Hullemen: Writing - original draft, Supervision, Conceptualization, Funding acquisition.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2019.10.062.

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