ABCB1, ABCG2 and PTEN determine the response of glioblastoma to temozolomide and ABT-888 therapy.

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Statement of translational relevance
Glioblastoma is a uniformly lethal disease and there is a great but yet unmet need for better therapies. There is a clear rationale for the clinical evaluation of PARP inhibitors such as ABT-888 (veliparib) in combination with the standard therapy in high-grade glioma patients. PARP inhibitors can augment the cytotoxic effects of DNA damage by interfering in DNA repair. Of all PARP inhibitors, ABT-888 is the clinically most advanced candidate agent for glioblastoma. Failure or success of these trials may not just determine the fate of ABT-888, but may set the stage for the whole concept of using PARP inhibitors in this disease. We here show that ABC-transporters at the blood-brain barrier and tumor cells and PTEN status of tumor cells are important determinants of efficacy of ABT-888 and temozolomide combination therapy. This information need to be taken into account during the evaluation of ongoing clinical trials.
ABSTRACT

**Purpose:** Little is known about the optimal clinical use of ABT-888 (veliparib) for treatment of glioblastoma. ABT-888 is a PARP inhibitor undergoing extensive clinical evaluation in glioblastoma, because it may synergize with the standard-of-care temozolomide (TMZ). We have elucidated important factors controlling ABT-888 efficacy in glioblastoma.

**Experimental design:** We used genetically engineered spontaneous glioblastoma mouse models and allograft models that were orthotopically transplanted into wildtype (WT) and Abcb1/Abcg2 deficient (KO) recipients. **Results:** ABT-888/TMZ is not efficacious against p53;p16<sup>ink4a</sup>/p19<sup>arf</sup>;K-Ras<sup>v12</sup>;LucR allografts in WT recipients, indicating inherent resistance. Abcb1/Abcg2 mediated efflux of ABT-888 at the blood-brain barrier (BBB) causes a 5-fold reduction of ABT-888 brain penetration (p<0.0001) that was fully reversible by elacridar. Efficacy studies in WT and KO recipients and/or concomitant elacridar demonstrate that Abcb1/Abcg2 at the BBB and in tumor cells impair TMZ/ABT-888 combination treatment efficacy. Elacridar also markedly improved TMZ/ABT-888 combination treatment in the spontaneous p53;p16<sup>ink4a</sup>/p19<sup>arf</sup>;K-Ras<sup>v12</sup>;LucR glioblastoma model. Importantly, ABT-888 does enhance TMZ efficacy in Pten deficient glioblastoma allografts and spontaneous tumors, even in Abcb1/Abcg2 proficient WT mice. Loss of PTEN occurs frequently in glioblastoma (36%) and *in silico* analysis on glioblastoma patient samples revealed that it is associated with a worse overall survival (310 days vs. 620 days, n=117). **Conclusions:** The potential of ABT-888 in glioblastoma can best be demonstrated in patients with PTEN null tumors. Therefore, clinical trials with ABT-888 should evaluate these patients as a separate group. Importantly, inhibition of ABCB1 and ABCG2 (by elacridar) may improve the efficacy of TMZ/ABT-888 therapy in all glioblastoma patients.
Glioblastoma (GBM) is the most common and aggressive primary brain tumor and only very few chemotherapeutic agents are available that exert a meaningful response. The current standard-of-care is surgical resection followed by chemo-radiation therapy consisting of the DNA-alkylating agent temozolomide (TMZ) and radiotherapy. This DNA damaging treatment modality significantly increases the overall median survival to 14.6 months after diagnosis (1). However, even with this aggressive treatment regimen the prognosis of GBM patients remains dismal and novel therapeutics are urgently needed.

Poly (ADP-ribose) polymerase (PARP) inhibitors enhance the activity of DNA damaging therapies, due to the critical function of PARP-1 and PARP-2 in base excision repair (2, 3). Several preclinical studies suggest that PARP inhibitors enhance the efficacy of TMZ in both sensitive and resistant tumors (4, 5). Moreover, their capacity to sensitize GBM cells to TMZ treatment and reverse TMZ resistance has been reported (6-8). ABT-888 (veliparib) is a potent PARP-1/2 inhibitor and the clinically most advanced candidate for glioblastoma, with several ongoing clinical trials. Obviously, the combination of ABT-888 with chemo-radiation therapy is receiving considerable interest, because it has shown promise in preclinical models, including intracranial models (9, 10). The outcome of these clinical trials may not only determine the fate of ABT-888, but may set the stage for the whole concept of using PARP inhibitors in the treatment of GBM. In order to maximize the chances for successful implementation of this drug, it is important to have a thorough understanding of the factors that may pose threats this objective. Because we felt that critical information was lacking, we have performed comprehensive in vivo studies in a set of clinically relevant glioma models to interrogate the potential of ABT-888 in high-grade glioma and identified critical factors that may determine its success in the clinic.
A pitfall of many intracranial tumor models, including those that were used to demonstrate efficacy of ABT-888 (9, 10), is the very leaky tumor vasculature, resulting in excellent drug penetration throughout the whole tumor area. However, due to the invasive nature of gliomas, the permeability of blood-brain barrier in such brain tumors (blood brain tumor barrier; BBTB) is much more heterogeneous in GBM patients and may therefore compromise adequate drug exposure to a substantial fraction of GBM cells (11-13). Especially agents that are recognized by ATP-binding cassette (ABC) drug efflux transporters expressed at the BBB are at risk. Of all efflux transporters present in the BBB, ABCB1 (also known as P-glycoprotein; P-gp or MDR1) and ABCG2 (Breast Cancer Resistance protein; BCRP) are dominant (14). Together, they are responsible for the efflux of a wide range of therapeutic agents, including many of the small molecule inhibitors that are currently under (clinical) investigation for brain cancer (15, 16). Moreover, ABCB1 and ABCG2 have established roles in conferring multidrug resistance by limiting intracellular drug accumulation in tumor cells (17, 18). Besides this issue of adequate drug exposure, many intracranial tumor models also lack many important characteristics, including those involved in drug resistance, as a consequence of culturing cells in serum-containing medium (19, 20). By ignoring these intrinsic and extrinsic drug resistance mechanisms, these models may not be predictive for the clinical efficacy of potential GBM therapeutics, such as ABT-888.

We have previously developed a range of Cre-LoxP conditional transgenic mouse models of GBM for chemotherapy intervention studies (21). These models allow interrogation of the influence of frequently altered genes in GBM (e.g. *P53* or *PTEN* status) on tumor sensitivity (22). In the present study, we found that a serum-free cultured cell line derived from a spontaneous *p53*;*p16*\textsuperscript{ink4a};*p19*\textsuperscript{Arf};*K-Ras*\textsuperscript{v12};*LucR* GBM and re-injected into nude mice did not respond to ABT-888 and temozolomide combination therapy. By using *Abcb1a1b* and/or *Abcg2* deficient recipient mice and the dual ABC-transporter inhibitor elacridar, we
demonstrate that these drug efflux transporters are causing resistance in this clinically relevant model. Moreover, we found that ABT-888 is more active in PTEN deficient tumors in our in vivo models.
METHODS

Drugs

ABT-888 was obtained from Selleck Chemicals (Houston, TX, USA), TMZ for in vitro experiments from Sigma-Aldrich (St Louis, MO, USA) and TMZ for in vivo studies from TEVA Pharma (Haarlem, The Netherlands). Elacridar was kindly provided by GlaxoSmithKline (Research Triangle Park, NC, USA) and zosuquidar by Eli Lilly (Indianapolis, IN, USA).

Animals

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. All experiments with animals were approved by the animal experiment committee of the institute. The animals used for pharmacokinetics studies were female wild-type (WT), Abcb1a/1b+/+, Abcg2+/+, and Abcb1a/1b−/−;Abcg2−/− mice of FVB genetic background, between 9 and 14 weeks of age. Pten;p16ink4a/p19arf;K-Rasv12;LucR and p53;p16ink4a/p19arf;K-Rasv12;LucR conditional mice were genotyped as described previously (21). Athymic (nude) WT and Abcb1a/1b−/−;Abcg2−/− mice of FVB background were used as recipient animals for orthotopic injection of neurosphere cultured GBM cells generated from the above mentioned conditional mouse models.

ABT-888 brain penetration

ABT-888 (10 mg/kg) was administered p.o. or i.v. and elacridar (100 mg/kg, p.o.) was given 15 min prior to ABT-888. Blood was collected by cardiac puncture or tail vein (for serial sampling). Brains were homogenized in 3 ml 1% (w/v) bovine serum albumin. ABT-888 plasma and brain samples were analyzed by LC-MS/MS as described (23) or for more
sensitive quantitation over a concentration range of 0.3-100 ng/mL, a modified LC-MS/MS system utilizing an ABI4000 mass detector (m/z 245.2 to 84.2 for ABT-888, and 248.2 to 87.2 for [D₃]-ABT-888). Pharmacokinetic parameters were calculated using PKSolver (24).

**Pharmacokinetic calculations and Statistical analysis**

Brain and plasma concentrations among multiple strains were compared by one-way analysis of variance (ANOVA) with Bonferroni post-hoc test. Survival fractions were calculated according to the Kaplan-Meier method using GraphPad Prism v6 (GraphPad Software, Inc., La Jolla, CA, USA). The log-rank test was used to compare survival of groups.

**Intracranial tumor models and bioluminescence imaging**

The procedures of stereotactic intracranial injection of lentivirus or tumor cells and bioluminescence imaging have been described in detail (21). In short, Pten; p16<sup>ink4a</sup>/p19<sup>Arf</sup>; K-Ras<sup>v12</sup>; LucR and p53; p16<sup>ink4a</sup>/p19<sup>Arf</sup>; K-Ras<sup>v12</sup>; LucR mice were injected intracranially with 2 µl of CMV-Cre lentivirus suspension 2 mm lateral and 1 mm anterior to the bregma, 3 mm below the skull. For orthotopic transplantation models, 2 µl of cell suspension containing 5,000 cells was injected in athymic WT and Abcb1a/1b<sup>-/-</sup>; Abcg2<sup>2/-</sup> nude mice. Tumor development was monitored by bioluminescence using the IVIS 200 (Perkin Elmer Inc., Waltham, MA, USA). BLI values were log-converted. Mean and SE BLI values of each cohort were calculated for each time point, until most animals within the cohort had to be sacrificed due to disease progression. Mice were sacrificed when clear neurological symptoms occurred or weight loss (≥ 20%) was observed.
Drug formulation and treatment regimen

ABT-888 (in DMSO:saline; 1:10) was administered orally at a dose of 10 mg/kg/ b.i.d. for 5 days. TMZ (100 mg capsule) was dissolved just prior to administration in 2 ml of ethanol plus 18 ml saline, filtered and administered orally at a dose of 100 mg/kg q.d x5 within 30 min after preparation. Elacridar was administered orally at a dose of 100 mg/kg 15 min prior to ABT-888 or TMZ administration.

GBM cell cultures and proliferation assays

GBM cell lines have been derived from tumors generated in p16\textsuperscript{lnk4a}/p19\textsuperscript{Arf};K-Ras\textsuperscript{v12};LucR, Pten;p16\textsuperscript{lnk4a}/p19\textsuperscript{Arf};K-Ras\textsuperscript{v12};LucR, p53;p16\textsuperscript{lnk4a}/p19\textsuperscript{Arf};K-Ras\textsuperscript{v12};LucR and p53;Pten;p16\textsuperscript{lnk4a}/p19\textsuperscript{Arf};K-Ras\textsuperscript{v12};LucR conditional mice after lentiviral infection. Small amounts of tumor tissue were triturated mechanically in ice-cold Ca\textsuperscript{2+} and Mg\textsuperscript{2+} free HBSS (Gibco, Carlsbad, CA, USA). Cell suspensions were cultured in ultra low binding 6-well plates (Corning, Corning, NY, USA) in serum-free MHM (Medium Hormone-Mix) medium supplemented with 20 ng/ml EGF and bFGF (Sigma) as described previously (25).

Proliferation assays were carried out with GBM cells (2,000/well) seeded on laminin coated 96-well black-well/clear-bottom plates (Greiner Bio-One, Alphen, The Netherlands) in MHM medium supplemented with 10 ng/ml EGF and bFGF. Treatment was started after 1 day with MHM medium containing 0.1% DMSO (control), or 100 µM TMZ alone, or in combination with increasing concentrations of ABT-888 (0.3 µM to 30 µM). Cell density/viability was determined on day 0 (treatment start) and day 5 using bioluminescence imaging on an IVIS Lumina II Imaging System (Perkin Elmer Inc.) with 150 µg/ml of beetle luciferin (Promega, Leiden, The Netherlands) in each well.
**Histology and Immunohistochemistry**

Brain tissue was fixed in 4% formaldehyde; paraffin embedded and cut into 4 µm coronal sections that were stained for H&E and for Abcg2/ABCG2 using the BXP-53 antibody (Abcam, Cambridge, UK).

**Western blotting**

For PARP inhibition analyses, GBM cells were cultured on laminin in 6-well plates until 80-90% cell confluency. Cells were incubated with drugs for 4 h and subsequently lysed with complete IPA buffer containing phosphatase inhibitors. Lysates were processed for Western blotting. Primary antibodies used in this study are rabbit anti-PAR (1:1000, Trevigen, Gaithersburg, MD, USA), mouse anti-β-tubulin isotype III (1:1000, Sigma, St Louis, MO, USA) and Mdr (H-19) rabbit polyclonal (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and BXP-53 rat monoclonal (1:400, Abcam, Cambridge, UK) for Abcb1 and Abcg2, respectively. Enhanced chemiluminescence (ECL) was used for detection using Molecular imaged ChemiDoc™ XRS+ system (Bio-Rad, Hercules, CA, USA). Data was analyzed using ImageLab software (version 2.0.1) from Bio-Rad laboratories.
RESULTS

p53; p16\textsuperscript{INK4a}/p19\textsuperscript{ARF}; K-Ras\textsuperscript{v12}; LucR GBM allografts are resistant to TMZ or ABT-888 and TMZ treatment

GBM652457 cells were isolated from a lentivirally induced tumor in a p53; p16\textsuperscript{INK4a}/p19\textsuperscript{ARF}; K-Ras\textsuperscript{v12}; LucR mouse. Tumors that developed after intracranial injection into nude mice resemble many features of human high-grade gliomas (21). Mice received either vehicle, single treatment with 100 mg/kg TMZ q.d. or combined with 10 mg/kg ABT-888 b.i.d. for 5 days. Although a slight delay in tumor growth was observed, no significant difference in median survival was found between treated and control (Fig. 1A; 11 vs. 11 days; \( p = 0.37 \)) or the two treatment groups (12 vs. 11 days; \( p = 0.22 \)).

Brain penetration of ABT-888 is restricted by Abcb1 and Abcg2 and can be enhanced by elacridar

We found that ABT-888 is transported by Abcb1, Abcg2 and ABCB1 in vitro (Supplementary Fig S1). Consequently, we evaluated the impact of Abcb1\textsuperscript{a/b} and/or Abcg2 on the plasma and brain pharmacokinetics of ABT-888 using WT, Abcb1\textsuperscript{a/b}\textsuperscript{+/-}, Abcg2\textsuperscript{+/-}, and Abcb1\textsuperscript{a/b}\textsuperscript{+/-}; Abcg2\textsuperscript{+/-} mice. Following i.v. administration of 10 mg/kg of ABT-888, the AUC\textsubscript{plasma} differed just 1.3-fold between the various strains (Table 1, Fig. 2A). Consequently, Abcb1 and Abcg2 do not play a critical role in the systemic clearance of ABT-888. In contrast, the AUC\textsubscript{brain} of both Abcb1\textsuperscript{a/b}\textsuperscript{+/-} and Abcb1\textsuperscript{a/b}\textsuperscript{+/-}; Abcg2\textsuperscript{+/-} mice was significantly higher than in WT mice (3.7 and 4.9-fold, respectively; both \( p < 0.0001 \)), demonstrating that Abcb1 and - to a lesser extent Abcg2 at the BBB severely impair the brain entry of ABT-888. Interestingly, a profound continuous increase in the ABT-888 brain-to-plasma ratio was observed in Abcb1\textsuperscript{a/b}\textsuperscript{+/-} and Abcb1\textsuperscript{a/b}\textsuperscript{+/-}; Abcg2\textsuperscript{+/-} mice in a time course of 4 hr. This implies that the
brain clearance does not keep up with the systemic clearance of ABT-888, demonstrating the importance of Abcb1 and Abcg2 in clearance of ABT-888 from the brain.

Likewise, inhibition of Abcb1 and Abcg2 by elacridar also increased ABT-888 brain accumulation. Concomitant elacridar slightly increased the AUC\textsubscript{plasma} by 1.2-fold (Table 1; \( p = 0.14 \)). This was also seen in \( Abcb1a/1b^{-/-};\text{Abcg2}^{2/-} \) mice (1.3-fold; \( p = 0.0099 \)) and thus most likely due to inhibition other drug elimination pathways. Importantly, co-administration of elacridar in WT mice caused an 11-fold increased ABT-888 brain concentration (\( p < 0.0001 \)) at 4 h after drug administration and a brain concentration-to-AUC\textsubscript{plasma} ratio similar to that of \( Abcb1a/1b^{-/-};\text{Abcg2}^{2/-} \) mice (with or without elacridar (\( p = 0.25 \) and \( p > 0.99 \) respectively)) and 9-fold higher than WT controls (\( p < 0.0001 \); Fig. 2B). Thus, elacridar significantly enhances the brain accumulation of ABT-888 by inhibition of Abcb1 and Abcg2 at the BBB.

**Co-administration of elacridar enhances the efficacy of TMZ and ABT-888 against GBM**

To investigate whether absence of Abcb1 and Abcg2 at the BBB/BBTB would improve TMZ/ABT-888 treatment, we repeated the \textit{in vivo} efficacy experiment with \( p53;\text{p16}^{-/-};\text{p19}^{-/-};\text{K-Ras}^{v12};\text{LucR} \) GBM652457 cells in \( Abcb1a/1b^{-/-};\text{Abcg2}^{2/-} \) nude mice. We observed a better overall survival with TMZ/ABT-888 treatment in \( Abcb1a/1b^{-/-};\text{Abcg2}^{2/-} \) mice (Fig. 1B) than in WT mice (Fig. 1A). Mice receiving ABT-888+TMZ survived significantly longer than untreated controls (15 vs. 9 days; \( p = 0.004 \)) whereas mice receiving TMZ alone did not (11 vs. 9 days; \( p = 0.30 \)).

Besides the presence of Abcb1 and Abcg2 at the BBB/BBTB, their expression in tumor cells may also impair the intracellular accumulation and efficacy of ABT-888. Many of the murine derived GBM cell lines, including GBM652457 express Abcg2 and also some Abcb1 and immunochemistry of Abcg2 revealed its presence in blood vessels and tumor cells (Fig. 3). As assessed by western blotting, ABT-888 has PARP inhibitory activity in the sub-
micromolar range in our GBM cell lines in vitro, which was modestly enhanced by elacridar (Supplementary Fig. S2). To investigate whether elacridar can improve the efficacy of TMZ and ABT-888 in vivo, we administered TMZ and ABT-888 with or without elacridar to both WT and Abcb1a/1b<sup>−/−</sup>;Abcg2<sup>−/−</sup> mice bearing orthotopic p53<sup>−/−</sup>;p16<sup> Ink4a</sup>/p19<sup>Arf</sup>;K-Ras<sup> v12</sup>;LucR GBM652457. Abcb1a/1b<sup>−/−</sup>;Abcg2<sup>−/−</sup> mice receiving elacridar survived significantly longer than Abcb1a/1b<sup>−/−</sup>;Abcg2<sup>−/−</sup> mice not receiving elacridar (Fig. 1C, median survival 26.5 vs. 22 days; p = 0.025). WT mice receiving elacridar in combination with TMZ and ABT-888 responded similarly as Abcb1a/1b<sup>−/−</sup>;Abcg2<sup>−/−</sup> mice receiving the combination. Elacridar significantly prolonged the survival of WT mice receiving TMZ and ABT-888 treatment (median survival 25 vs. 18 days for WT mice receiving TMZ+ABT-888+elacridar vs. TMZ+ABT-888 respectively; p = 0.039). Together, these results support the idea that elacridar inhibited Abcb1 and Abcg2 both at the BBB and in tumor cells.

As a second model, we used our spontaneous high-grade glioma model induced by lentivirus in LoxP conditional p53<sup>−/−</sup>;p16<sup> Ink4a</sup>/p19<sup>Arf</sup>;K-Ras<sup> v12</sup>;LucR mice (21) (Fig. 1D). TMZ alone has an effect on the survival in this model and elacridar enhances the efficacy of TMZ, because TMZ is a (weak) substrate of Abcb1 and Abcg2 (26). ABT-888 combined with TMZ only marginally improved median survival (25 vs. 21 days for TMZ+ABT-888 vs. TMZ respectively; p = 0.14). However, concomitant elacridar with TMZ+ABT-888 significantly improved median survival (33 vs. 25 days for TMZ+ABT-888+elacridar vs. TMZ+ABT-888 respectively; p = 0.023).

**PTEN deficiency renders GBM sensitive to TMZ and ABT-888 treatment in vitro and in vivo**

Deletion or inactivation of PTEN is a frequent event in GBM. By performing an in silico analysis on 117 GBM patient samples, we found that patients whose tumor is deficient in
PTEN have a significantly worse overall survival than patients whose tumors are proficient in PTEN, viz. 310 vs. 620 days; $p = 5.16 \times 10^{-6}$ (Fig. 4A). On the other hand, it was recently shown by in vitro experiments that PTEN-null astrocytes have a compromised homologous recombination DNA repair pathway, rendering these more sensitive to treatment with TMZ and the PARP inhibitor ABT-888 due to synthetic lethality (27). We have now used our GBM models to interrogate the role of PTEN in the in vivo response to TMZ and ABT-888 treatment. GBM cell lines from spontaneous tumors including genotypes with additional Pt en deletion demonstrate similar PARP inhibition by ABT-888 (Fig. S2). However, $Pten;16^{lkaa}/p19^{arf};K-Ras^{v12};LucR$ GBM cells were much more sensitive to TMZ+ABT-888 than $p53;16^{lkaa}/p19^{arf};K-Ras^{v12};LucR$ cells (Fig. 4B). Likewise, additional deletion of Pten sensitized P53 deficient cells to ABT-888, albeit not to the level as of P53 proficient cells.

We treated intracranial $Pten;16^{lkaa}/p19^{arf};K-Ras^{v12};LucR$ GBM established in WT and $Abcb1a/1b^{+/-};Abcg2^{+/-}$ mice with TMZ or TMZ+ABT-888 (Fig. 4C). The survival is short, due to the aggressive growth of these cells in vivo. In WT mice TMZ+ABT-888 treatment improved median survival relative to control group or TMZ treated animals (10, 8 and 7 days for TMZ+ABT-888, TMZ and controls, respectively; TMZ+ABT-888 vs. TMZ: $p = 0.044$; TMZ+ABT-888 vs. control: $p = 0.0004$). Note that such a therapeutic benefit of ABT-888 was not found in WT mice bearing $p53;16^{lkaa}/p19^{arf};K-Ras^{v12};LucR$ GBM (Fig. 1A). Importantly, $Abcb1a/1b^{+/-};Abcg2^{+/-}$ mice benefitted even more from treatment (median survival: 12 vs. 9 days for KO mice receiving TMZ+ABT-888 vs. TMZ respectively. $p = 0.0006$). Subsequently, we evaluated the effect of PTEN deficiency in our lentivirally induced spontaneous $Pten;16^{lkaa}/p19^{arf};K-Ras^{v12};LucR$ GBM model. Whereas the treatment effect in the $P53;16^{lkaa}/p19^{arf};K-Ras^{v12};LucR$ GBM model generally becomes apparent not before some days after completion of the 5-day treatment, a marked effect in the spontaneous PTEN deficient tumors was already observed between days 0 and 7.
(Fig. 4D). Although there appears to be a trend toward a more accelerated growth later on, the median survival of mice receiving TMZ and ABT-888 combination treatment relative to TMZ alone increased significantly (19 vs. 15 days; $p = 0.0045$) while TMZ treatment alone did not affect median survival compared to vehicle control (15 vs. 14 days; $p = 0.15$).
DISCUSSION

This study, using clinically relevant *in vivo* high grade glioma mouse models, shows that the PARP inhibitor ABT-888 can improve the efficacy of TMZ chemotherapy against high-grade gliomas, but also identified two important factors that need to be considered when analyzing the clinical trials with ABT-888 in GBM. Firstly, ABCB1 and ABCG2 restrict both BBB penetration and tumor cell entry of ABT-888. Secondly, therapeutic benefit of ABT-888 added to TMZ in ABC-transporter proficient WT mice was only significant in *PTEN* deficient tumors. Reassuringly, responses were observed at a dose level of ABT-888 yielding clinically relevant systemic exposure. Based on these results, we propose that the *PTEN* status of the tumor should be taken into account during the analysis of the clinical trials. Moreover, concomitant inhibition of ABCB1 and ABCG2 by elacridar may further improve the efficacy of ABT-888+TMZ combination treatment, so that *PTEN* proficient tumors may also become responsive and *PTEN* deficient tumors are sensitized even further.

*P53*, *PTEN* and *P16^{loxp}/P19^{Arf}* are among the most frequently mutated or deleted genes in GBM (22). We have previously developed high-grade glioma models using loxP conditional transgenic mice that are deleted in brain cells following intracranial injection of Lenti-Cre virus. These spontaneous tumors, as well as tumors from neurosphere cultured cell lines derived of these and reinjected into recipient mice, resemble many features that are characteristic for human GBM (21). In the present study, we have used these models to evaluate the efficacy of ABT-888 to potentiate the activity of TMZ against high-grade glioma. Notably, the first study using WT mice injected with *P53;Ink4a/Arf;K-Ras^{v12}* GBM652457 cells demonstrated only a very minor response to this combination therapy. We demonstrate that ABT-888 is a good substrate of the drug efflux transporters Abcb1 and Abcg2 and
observed a more favorable response against GBM652457 cells injected into Abcb1a/1b\(^{-/-}\);Abcg2\(^{-/-}\) mice. Abcg2 was present in blood vessels of ABC-transporter WT mice (Fig. 3), but not in blood vessels of Abcg2 deficient Abcb1a/1b\(^{-/-}\);Abcg2\(^{-/-}\) mice. The expression of ABCB1 in the tumor vessels of gliomas in patients is well documented (28-30). Likewise, we were able to demonstrate the presence of ABCG2 in tumor vessels of patient specimens. Importantly, both in humans and in mice, ABCG2/Abcg2 was also found in tumor cells. Just recently, expression of ABCG2 in GBM was also demonstrated by Bahtia et al (31), who reported nuclear localization of ABCG2 in a subpopulation of GBM cells. A similar pattern was also seen in some of our patient specimens (Fig 3C). Abcg2 staining was not uniform throughout the tumor. Considering that ABCG2 is a marker of early progenitor or stem cells, these Abcg2 positive regions may reflect the areas of the tumor enriched for tumor initiating cells. The presence of ABCB1 in GBM tumor cells is still debatable (32). However, we successfully used elacridar in combination with TMZ and ABT-888 to further increase the efficacy in tumors grown in Abcb1a/1b\(^{-/-}\);Abcg2\(^{-/-}\) recipient mice, which indicates that drug transporters in these cells form a secondary barrier, as schematically depicted in Fig. 5.

Clearly, the response to ABT-888 that is observed in our high-grade glioma models is not as profound as reported previously (9, 10) using other intracranial models. There are several reasons that may explain this discrepancy. First, our models have more tight BBTB properties, including the expression of ABC drug transporters, whereas the intracranial models in previously reported studies harbor leaky vessels, which may lead to over-prediction of their efficacy.

Secondly, the GBM cell lines used for the classical models have been maintained on serum containing media for many generations, which is known to cause loss of critical characteristics conserved within tumor stem cells. Importantly, these include chemotherapy resistance profiles such as expression of ABCG2, active DNA-repair capacity, and resistance
to apoptosis (33, 34). Most likely, the high-grade models (allograft and spontaneous) used in this study are much more stringent models for drug testing.

Finally, we used a dose of 10 mg/kg throughout all in vivo studies since this dose results in a clinically relevant systemic exposure of ABT-888. Recent clinical trials report a peak plasma level of 500 ng/ml with an AUC of 3500 ng/ml*h (35). Previous preclinical studies have been using much higher doses of up to 50 mg/kg. Further increasing the dose in patients to improve efficacy, may result in unacceptable toxicity. Interestingly, co-administration of elacridar hardly affected the systemic exposure of ABT-888 (Fig 2), but enhanced the brain concentration of ABT-888 by 11-fold at 4 h. Consequently, this combination may enable improved local drug delivery to the brain, without increasing systemic exposure and possibly toxicity of ABT-888.

When exploring the efficacy of ABT-888 in our panel of spontaneous high-grade glioma derived cell lines, we also found that cell lines that are deficient in PTEN were much more sensitive to TMZ and ABT-888 treatment. Our results are in line with the in vitro data showing that PTEN-null astrocytes have a disturbance in the homologous recombination DNA repair pathway causing synthetic lethality when base excision repair activity is inhibited by a PARP inhibitor (27). Next, we took advantage of the fact that we could further explore the clinical relevance of this finding using our in vivo high-grade glioma models. Both WT and Abcb1a/1b−/−;Abcg2−/− mice bearing tumors from Pten;Ink4a/Arf;K-Rasv12 GBM696677 responded better to the TMZ and ABT-888 treatment than the mice bearing P53-null;PTEN proficient tumors. Notably, however, although the PTEN deficient GBM696677 cell line is more sensitive to TMZ and ABT-888 treatment, the effect of this combination on the tumor growth in vivo was not as dramatic as might have been hoped for based on the results obtained in vitro. This finding on one hand reminds us that the tumor micro-environment of GBM cells proliferating in vivo can help these cells to escape from lethal drug effects. On the other hand,
it highlights the importance of using the appropriate preclinical models to evaluate drug efficacy.

We expect that these preclinical findings will be useful to interpret the outcome of the currently ongoing clinical trials with ABT-888 in glioma patients (ClinicalTrials.gov identifiers: NCT01026493, NCT01514201, NCT00770471). In particular our data call for a subgroup analysis in GBM patients with PTEN deletion. PTEN loss is a frequent event in GBM (36% of cases). Notably, as assessed by an in silico analysis performed on 117 glioblastoma patients, PTEN loss is associated with a more dismal prognosis but our study provides reasonable evidence that this subgroup of patients may benefit most from ABT-888. This benefit, however, may get lost when analyzing all GBMs as one group. Furthermore, we have shown that the efficacy of ABT-888 and TMZ combination therapy is attenuated by ABCB1 and ABCG2, supporting the initiation of clinical trials investigating the potential of ABC-transporter inhibitors (e.g. elacridar) in glioma therapy.

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Reference List


FIGURE LEGENDS

Fig. 1. Inhibition of Abcb1 and Abcg2 improve efficacy of ABT-888 + TMZ treatment.

(A) Efficacy of TMZ vs TMZ+ABT-888 treatment against intracranial p53;16\textsuperscript{Inkda}/p19\textsuperscript{Arf};K-Ras\textsuperscript{v12};LucR GBM652457 cells injected into WT mice. (B) Same setup but now injected into Abcb1a/1b\textsuperscript{-/-};Abcg2\textsuperscript{-/-} (KO) mice. (C) Efficacy of TMZ+ABT-888 with and without elacridar in both WT and KO mice. (D) Efficacy of TMZ or TMZ+ABT-888 with or without elacridar against (lentivirally induced) spontaneous p53;16\textsuperscript{Inkda}/p19\textsuperscript{Arf};K-Ras\textsuperscript{v12};LucR tumors. TMZ p.o. at 100 mg/kg qd. alone or concurrently with ABT-888 p.o. at 10 mg/kg bid. and/or elacridar p.o. at 100 mg/kg qd. 15 min prior to TMZ for 5 days. Left panels, relative tumor growth curves (bioluminescence imaging; means ± SEM). Right panels, Kaplan-Meier analysis of survival. Statistical significance was determined by Log-rank test. A: n= 5, 6 and 6 WT mice in control, TMZ and TMZ+ABT-888 treated groups, respectively; B, n= 9, 6 and 7 KO mice in control, TMZ and TMZ+ABT-888 groups respectively. C, n=3, 5, 6, 8, 8 for control, TMZ+ABT-888 (WT), TMZ+ABT-888+elacridar (WT) and both KO groups respectively. D, n=17, 19, 22, 11, 14 for control, TMZ, TMZ+elacridar, TMZ+ABT-888 and TMZ+ABT-888+elacridar groups respectively.

Fig. 2. The brain penetration of ABT-888 is limited by Abcb1 and Abcg2.

(A) ABT-888 plasma concentrations, brain concentrations and brain-to-plasma ratios following i.v. administration of 10 mg/kg of ABT-888 (n=5/time point/strain). B, ABT-888 levels following 10 mg/kg p.o. administered to WT and Abcb1a/1b\textsuperscript{-/-};Abcg2\textsuperscript{-/-} mice with/without co-administration of 100 mg/kg elacridar p.o.. Blood samples were collected from the tail at 15 min, 1, 2 and 4 hr (n=8/strain). Brain samples were harvested at 4 hr after
drug administration. Data are presented as means ± SEM, **** p < 0.0001 compared to WT. ANOVA followed by Bonferroni post-hoc analysis.

Fig. 3. Bcrp1/BCRP is present at the BBB/BBTB and also expressed in tumor cells.

Bcrp1 staining in p53;p16<sup>ink4a</sup>/p19<sup>arf</sup>;K-Ras<sup>v12</sup>;LucR GBM652457 tumors in WT (A) and Abcb1a/1b<sup>−/−</sup>;Abcg2<sup>−/−</sup> nude mice (B). In WT mice Abcg2 is expressed at the BBB, in blood vessels throughout the tumor (BBTB) as well as on tumor cells. The presence of Abcg2 in tumor cells is even better visualized in the Abcg2 deficient Abcb1a/1b<sup>−/−</sup>;Abcg2<sup>−/−</sup> recipients. Notably, the expression in tumor cells is not uniform throughout the tumor. C, Similar ABCG2 staining was observed in vessels and tumor cells in human GBM samples as depicted in these two examples. (Bar size: 2 mm (A,B left) and 200 um (all other panels). D. Western blot analysis demonstrates the presence of Abcb1 and Abcg2 in GBM cell lines of different origins: p53;p16<sup>ink4a</sup>/p19<sup>arf</sup>;K-Ras<sup>v12</sup>;LucR (GBM652457, GBM910300), Pten;p16<sup>ink4a</sup>/p19<sup>arf</sup>;K-Ras<sup>v12</sup>;LucR (GBM696677), P53;Pten;p16<sup>ink4a</sup>/p19<sup>arf</sup>;K-Ras<sup>v12</sup>;LucR (GBM707263, GBM707251) and p16<sup>ink4a</sup>/p19<sup>arf</sup>;K-Ras<sup>v12</sup>;LucR (GBM763663). Control cell lines: CT26 is a non-drug selected murine colon cancer and K1735 is a non-drug selected murine melanoma cell line, both with endogenous expression of Abcb1, K1735-Taxol was drug-selected by stepwise increasing paclitaxel concentrations up to 0.5 µM, MDCK-Bcrp1 (murine Abcg2 transduced) and MDCK-parent (non-transduced) Madine-Darby Canine Kidney cells.

Fig. 4. PTEN deficient tumors are more sensitive to ABT-888 + TMZ treatment.

(A) Kaplan-Meier analysis of PTEN deletion (≤ 1.8 copies) on overall survival of GBM patients (REMBRANDT database (36, 37)). (B) Sensitivity (in vitro) of two panels of GBM cell lines of different genetic backgrounds exposed to 100 µM TMZ and increasing
concentrations of ABT-888 for 5 days. Data (mean ± SEM; n=4) growth percentage relative to treatment with TMZ alone. (C) Efficacy of ABT-888 in combination with TMZ against Pten;p16^{ink4a}/p19^{arf},K-Ras^{v12};LucR GBM696677 cells injected intracranially into WT or Abcb1a/1b^{-/-};Abcg2^{-/-} (KO) mice and (D) (lentivirally induced) spontaneous Pten;p16^{ink4a}/p19^{arf},K-Ras^{v12};LucR tumors. TMZ (100 mg/kg p.o. qd) alone or concurrently with ABT-888 (10 mg/kg p.o. bid) for 5 days. C and D left panels, relative tumor growth curves (means ± SEM). C and D right panels, Kaplan-Meier analysis of survival. Statistical significance was determined by Log-rank test. C, n= 8, 8, 10, 9 for control, WT, TMZ (KO) and TMZ+ABT-888 (KO) groups respectively. D, n= 13, 12, 11 for control, TMZ and TMZ+ABT-888 groups respectively.

**Fig. 5. ABCB1/ABCG2 at both the BBB/BBTB and in tumor cells limit drug entry and efficacy.**

A-C depicts the situation in WT mice, where drug penetration into tumors is higher when elacridar inhibits ABCB1/ABCG2 at the BBB/BBTB and in tumor cells and drug retention is prolonged while systemic clearance has taken place (C). Drug penetration is higher in Abcb1a/1b^{-/-};Abcg2^{-/-} (KO) mice (D) but the best efficacy is achieved when Abcb1/Abcg2 in tumor cells is inhibited by elacridar (E).

**Table 1. Plasma and Brain AUC of ABT-888.**

WT, Abcb1a/b^{-/-}, Abcg2^{-/-} and Abcb1a/b^{-/-};Abcg2^{-/-} mice received i.v. or p.o. ABT-888 (10 mg/kg). AUC(0-4h) in ng/ml*h or ng/g*h (mean ± SEM). ** p < 0.01, **** p < 0.0001 compared to WT mice. #### p < 0.0001 compared to Abcb1a/b^{-/-}. + p < 0.05, ++ p < 0.01, compared to Abcb1a/b;Abcg2^{-/-} mice not receiving elacridar. One way ANOVA with Bonferroni posthoc test.
Figure 1

A

B

C

D
Figure 2
Figure 3

A

B

C

D

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Abcg2
Figure 4

A

PTEN del vs. WT: \( p = 5.16 \times 10^{-6} \)
PTEN del vs. all GBM: \( p = 1.08 \times 10^{-6} \)

- all GBM
- PTEN del
- PTEN WT

B

Cell growth (%)

[ABT] (µM) in presence of TMZ

C

Tumor growth (%)

Survival (%)

D
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<th>AUC&lt;sub&gt;0-4h&lt;/sub&gt;</th>
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ABCB1, ABCG2 and PTEN determine the response of glioblastoma to temozolomide and ABT-888 therapy

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