

## ORIGINAL ARTICLE

# The pyridinylfuranopyrimidine inhibitor, PI-103, chemosensitizes glioblastoma cells for apoptosis by inhibiting DNA repair

M-A Westhoff<sup>1,5</sup>, JA Kandenwein<sup>1,2,5</sup>, S Karl<sup>1</sup>, SHK Vellanki<sup>1</sup>, V Braun<sup>3</sup>, A Eramo<sup>4</sup>, G Antoniadis<sup>1</sup>, K-M Debatin<sup>1</sup> and S Fulda<sup>1</sup>

<sup>1</sup>University Children's Hospital, Ulm, Germany; <sup>2</sup>Department of Neurosurgery, University of Ulm, Bezirkskrankenhaus Guenzburg, Germany; <sup>3</sup>Department of Neurosurgery, Evangelisches Jung-Stilling-Krankenhaus, Siegen, Germany and <sup>4</sup>Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanita, Rome, Italy

**The failure of conventional therapies in glioblastoma (GBM) is largely due to an aberrant activity of survival cascades, such as PI3 kinase (PI3K)/Akt-mediated signaling. This study is the first to show that the class I PI3K inhibitor, PI-103, enhances chemotherapy-induced cell death of GBM cells. Concurrent treatment with PI-103 and DNA-damaging drugs, in particular doxorubicin, significantly increases apoptosis and reduces colony formation compared with chemotherapy treatment alone. The underlying molecular mechanism for this chemosensitization was shown by two independent approaches, that is, pharmacological and genetic inhibition of PI3K, DNA-PK and mTOR, to involve inhibition of DNA-PK-mediated DNA repair. Accordingly, blockage of PI3K or DNA-PK, but not of mTOR, significantly delays the resolution of doxorubicin-induced DNA damage and concomitantly increases apoptosis. Importantly, not only are several GBM cell lines chemosensitized by PI-103 but also GBM stem cells. Clinical relevance was further confirmed by the use of primary cultured GBM cells, which also exhibit increased cell death and reduced colony formation on combined treatment with PI-103 and doxorubicin. By identifying class I PI3K inhibitors as powerful agents in enhancing the lethality of DNA-damaging drugs, to which GBMs are usually considered unresponsive, our findings have important implications for the design of rational combination regimens in overcoming the frequent chemoresistance of GBM.**

*Oncogene* (2009) 28, 3586–3596; doi:10.1038/onc.2009.215; published online 27 July 2009

**Keywords:** apoptosis; PI3 kinase; GBM

## Introduction

Glioblastoma (GBM), the most common type of malignant brain tumor, is an extremely difficult-to-treat

disease with high lethality (DeAngelis, 2001). Although the care of GBM patients is usually multidisciplinary and includes surgery, radiotherapy and chemotherapy (Stupp *et al.*, 2006), current treatment regimens have so far failed to significantly increase median survival after diagnosis, which is <12 months (Ohgaki *et al.*, 2004). This highlights the need for novel and improved therapeutic approaches. At present, the hope to achieve such an improvement lies within combining different treatment regimes; for example, currently, radiotherapy is frequently combined with temozolomide (Newton, 2008). This seems to be of particular importance in GBM, as these tumors have evolved alternating mechanisms of treatment resistance so that a monotherapy might not target the whole tumor cell population (Cavaliere *et al.*, 2007). To design new, successful strategies, a precise knowledge of tumor-specific alterations is needed, and one promising target for such an approach in GBM is the PI3K/Akt pathway (Colman and Aldape, 2008; Maira *et al.*, 2008).

Alterations in this signaling network have a pivotal role in a large range of tumors, including GBM (Cancer Genome Atlas Research Network, 2008; Parsons *et al.*, 2008), in which a correlation between the activity of this pathway and adverse clinical outcome and reduced apoptosis was shown (Chakravarti *et al.*, 2004). Furthermore, we previously showed that inhibition of PI3K sensitizes GBM to chemotherapy- and to death receptor-induced apoptosis (Opel *et al.*, 2008). A similar importance of the PI3K/Akt pathway has also been reported in ovarian, gastric, breast and lung cancer (Jiang and Liu, 2008), as well as in neuroblastoma, for which it was recently shown that aberrant activation of Akt correlates with reduced life expectancy (Opel *et al.*, 2007).

The PI3K network is a highly complex, multi-armed signaling machinery, which can activate numerous downstream effectors (Maira *et al.*, 2008). Of particular interest in this context is the interaction of this signaling network and anticancer drugs, as many chemotherapeutics act by damaging, directly or indirectly, DNA, which in turn leads to the activation of damage-response pathways (Christmann *et al.*, 2003). For example, the nonhomologous end-joining (NHEJ) repair machinery is activated on DNA double-strand breaks (DSBs)

Correspondence: Professor Dr S Fulda, University Children's Hospital, Eythstrasse 24, D-89075 Ulm, Germany.  
E-mail: simone.fulda@uniklinik-ulm.de

<sup>5</sup>These authors contributed equally to this work.

Received 22 February 2009; revised 23 May 2009; accepted 1 June 2009; published online 27 July 2009

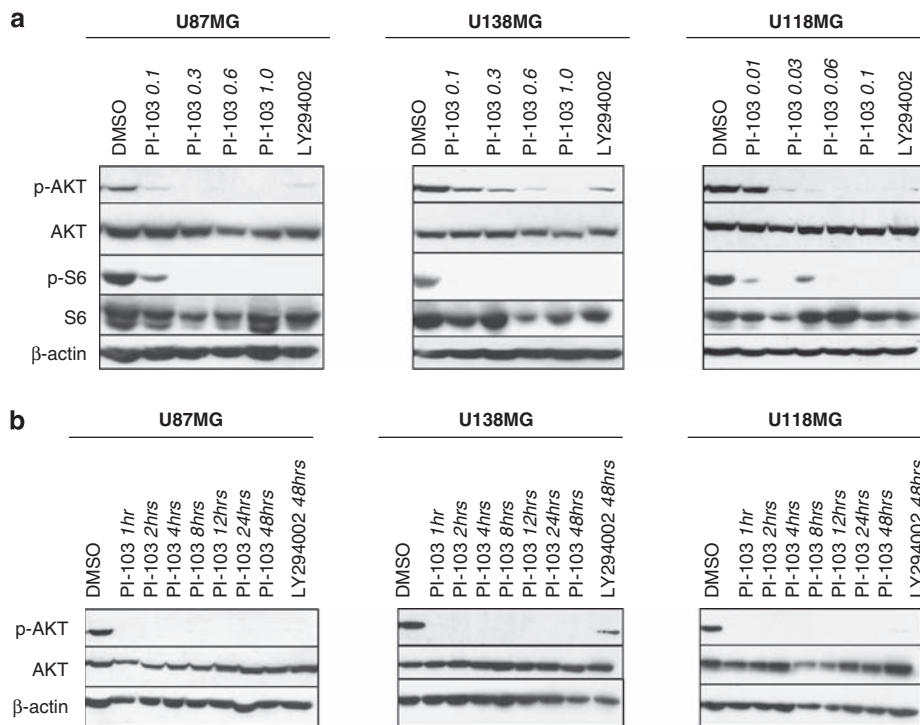
(Christmann *et al.*, 2003), and many proteins involved in this repair pathway are genetically and structurally similar to PI3K (Fruman *et al.*, 1998), such as ATM and ATR proteins and probably most strikingly DNA-PK (Jackson, 2002). The latter protein is responsible for ‘cleaning up’ the DNA ends at break point, a process essential for repair initiation (Jackson, 2002). Furthermore, DNA-PK has been shown to be a co-activator of Akt (Bozulic *et al.*, 2008), but the inverse relationship between these two kinases, with Akt being an activator of DNA-PK, has also been described (Toulany *et al.*, 2008). This suggests that the PI3K/Akt pathway and DNA repair processes are intricately linked.

Currently, there is much interest in exploiting the PI3K/Akt pathway for anticancer drug discovery and a series of small-molecule inhibitors have been developed (Yap *et al.*, 2008; Ihle and Powis, 2009; Maira *et al.*, 2009). For example, class I PI3K inhibitors, such as the pyridinylfuranopyrimidine inhibitor PI-103, are considered as promising candidates for such an approach (Ihle and Powis, 2009; Maira *et al.*, 2009). Although PI-103 has been shown to block proliferation (Fan *et al.*, 2006, 2007) and to enhance the efficacy of radiotherapy (Prevo *et al.*, 2008), its potential as a chemosensitizer has so far not been addressed. Therefore, in this study, we investigated the effect of PI-103 in combination with DNA-damaging drugs in GBM.

## Results

### PI-103 inhibits PI3K-mediated signaling in GBM cells

To find the ideal conditions under which the class I PI3K inhibitor, PI-103, blocks PI3K-mediated signaling in GBM cells, we initially determined the optimal dose and temporal efficacy of PI-103. Inhibition of PI3K was assayed indirectly, using the phosphorylation of Akt and S6 ribosomal protein as surrogate readouts for the activity of PI3K and its mTOR-dependent sidearm, respectively. For this purpose, three prototypical GBM cell lines were chosen, U87MG, U118MG and U138MG, all of which harbor PTEN mutations (Ishii *et al.*, 1999) and thus express relatively high levels of constitutively (that is, even in the absence of serum-based growth factors) phosphorylated, hence active, Akt, whereas U138MG and U118MG cell lines express mutant p53 protein, which is wild type in U87MG cells (Ishii *et al.*, 1999). As shown in Figure 1, PI-103 inhibits PI3K signaling at low micromolar concentrations at least as efficiently as does LY294002, a broad-range inhibitor of the PI3K family used as a positive control throughout this study, at a much higher concentration of 20  $\mu\text{M}$ . This holds true for a range of different concentrations of PI-103 at a single time point (Figure 1a), as well as for a single concentration over a prolonged period of time (Figure 1b). We observe an



**Figure 1** Inhibition of the PI3K/Akt signaling cascade by PI-103. (a) Cells were treated for 24 h with solvent (dimethylsulfoxide (DMSO)), increasing concentrations of PI-103 (indicated in  $\mu\text{M}$ ) or 20  $\mu\text{M}$  of LY294002, or (b) with a defined concentration of PI-103 (U87MG: 0.6  $\mu\text{M}$ ; U138MG: 1.0  $\mu\text{M}$ ; U118MG: 0.06  $\mu\text{M}$ ) for indicated times (in h) or with 20  $\mu\text{M}$  of LY294002 for 48 h. Protein expression levels and phosphorylation status of Akt, S6 ribosomal protein and  $\beta$ -actin were analyzed by western blotting. A representative result of at least three independent experiments is shown.

~15-fold variation in cell line-specific sensitivity to the PI-103, a range which is similar to that which has been antecedently described (Chen *et al.*, 2008).

#### *PI-103 sensitizes GBM cells for cytotoxic drug-induced apoptosis*

After having established the concentrations of PI-103 in the inhibition of PI3K-mediated signaling in GBM cells, we treated the GBM cell lines with DNA-damaging drugs (Figure 2a) in the presence of sub-toxic concentrations of PI-103 (Supplementary Figure 1a). We selected the topoisomerase II inhibitor and DNA-intercalating agent, doxorubicin, the topoisomerase II inhibitor, etoposide (Nitiss, 2002), and the alkylating agent, temozolomide (Tisdale, 1985). Importantly, PI-103 significantly enhanced cytotoxic drug-induced apoptosis in all cell lines to a drug- and cell line-specific degree (Figures 2a–c). For U87MG and U138MG cell lines, this sensitization effect was synergistic for PI-103, combined with either doxorubicin or etoposide (Supplementary Table 1). In contrast, U118MG cells could not be sensitized for doxorubicin-induced death, as these cells were resistant to doxorubicin even after prolonged treatment (Supplementary Figure 1b). It is noteworthy that sensitization to doxorubicin- or etoposide-induced apoptosis by PI-103 occurred independently of treatment schedule, as pre-incubation with PI-103 proved to be of no advantage compared with concurrent treatment (Supplementary Figures 1c and d). LY294002 seems to be more effective in sensitizing U87MG and U138MG cells after doxorubicin treatment, whereas PI-103 seems to be more potent in combination with etoposide (Figures 2a and b). This difference might be due to a broader inhibition of PI3K-like kinases, for example, DNA-PK, by LY294002 (Yaneva *et al.*, 2005). As these PI3K-like kinases are frequently associated with DNA damage, which not only occurs on topoisomerase II treatment but also when doxorubicin intercalates into DNA (Nitiss, 2002), this might explain the perceived differences between LY294002 and PI-103.

To exclude the hypothesis that sensitization for cytotoxic drug-induced apoptosis by PI-103 is just a short-term effect, we performed colony assays to assess long-term survival. To this end, U87MG cells were seeded at low density and treated for 3 h with doxorubicin in the presence or absence of PI-103, and then left to grow for 10 days. Importantly, the combined treatment with doxorubicin and PI-103 led to a significantly stronger reduction in colony formation than did treatment with doxorubicin alone (Figure 2d).

Next, we wanted to know whether PI-103 also increases cell death in GBM stem cells, as these cells have been shown to be highly resistant to radio- and chemotherapy (Bao *et al.*, 2006; Eramo *et al.*, 2006). To address this question, we used GBM stem cells that we previously characterized (Eramo *et al.*, 2006). Crucially, combining PI-103 and doxorubicin significantly reduced cell viability of GBM stem cells when compared with treatment with either doxorubicin or PI-103 alone (Figure 3a).

To further verify the clinical relevance of our findings, we also used primary cultured GBM cells generated from surgical specimens (Opel *et al.*, 2008). PI3K signaling was inhibited by PI-103 in these cells at similar concentrations to those used in GBM cell lines (Supplementary Figure 2a). Importantly, PI-103 significantly increased doxorubicin- and etoposide-induced apoptosis in these primary cultured GBM cells (Figure 3b). Although a trend toward sensitization could also be observed when temozolomide was combined with PI-103, this sensitization effect was only significant for LY294002 (Figure 3b). Further, the combination treatment of PI-103 and doxorubicin was significantly more efficient in suppressing colony formation than was either treatment alone (Figure 3c), showing that the combination treatment also has a long-term effect on primary cultured GBM cells.

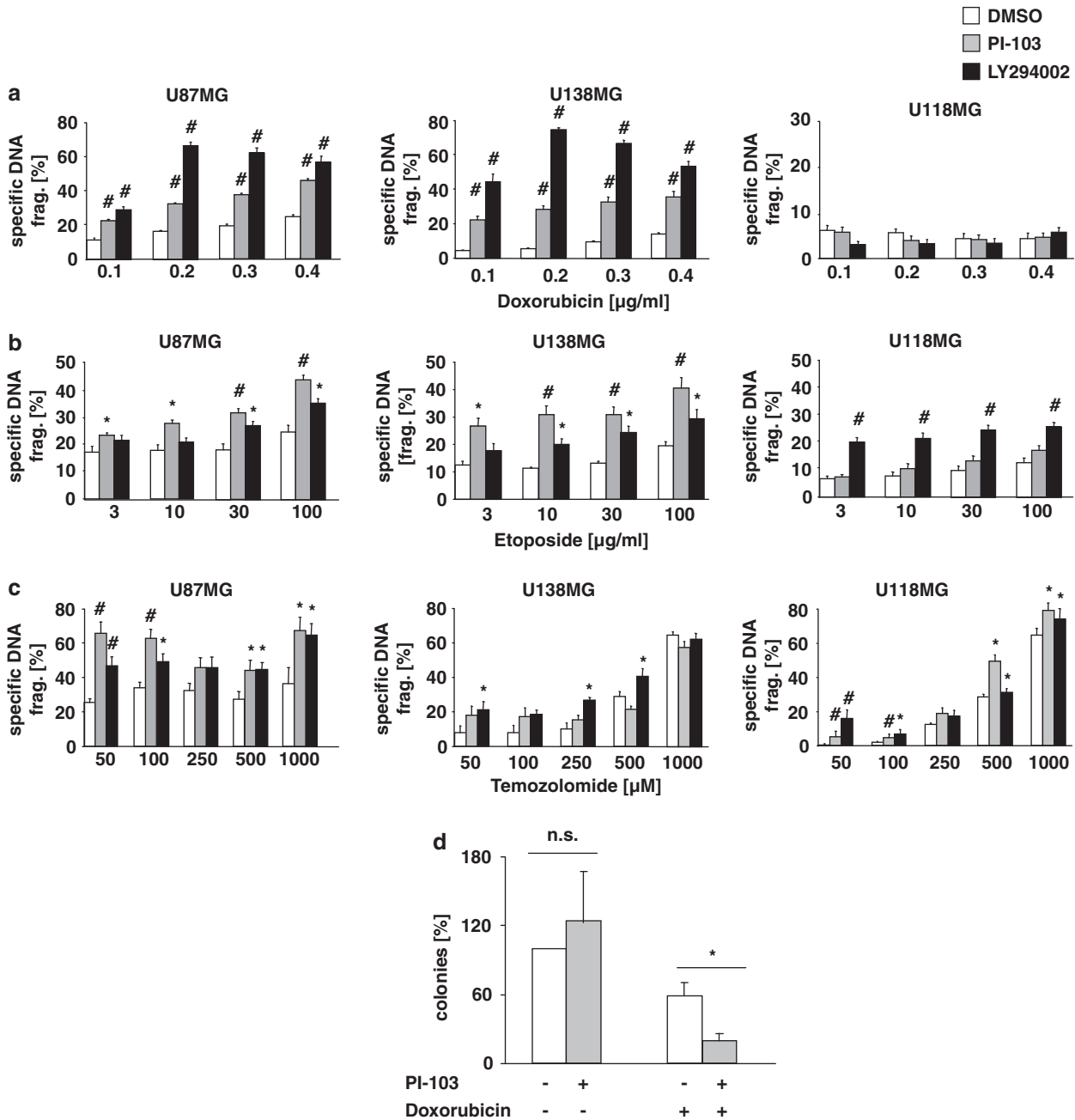
This set of experiments shows that PI-103 sensitizes GBM cells—cell lines, GBM stem cells and primary material—for apoptosis induced by DNA-damaging drugs, such as doxorubicin, etoposide and temozolomide. Furthermore, this sensitization is not just a short-term phenomenon, as shown by the long-term suppressive effects of even a brief treatment of PI-103 in combination with doxorubicin on colony formation, compared with treatment with doxorubicin alone.

#### *PI-103 prolongs doxorubicin-induced DNA damage and retards clearance of $\gamma$ H2A.X foci*

As interference with PI3K-mediated signaling can alter cellular response to DNA damage, we hypothesized that the chemosensitizing effect of PI-103 on treatment with DNA-damaging drugs might affect the cell at the level of DNA damage/repair. As PI-103 showed a strong and consistent sensitization effect when combined with DSB-inducing agents, that is, topoisomerase II inhibitors doxorubicin and etoposide, we assessed DNA damage induced by either drug in the absence and presence of PI-103. For this, we used alkaline comet assay, the readout of which, referred to as Olive Tail Moment, is considered to correlate directly with the amount of DNA damage (Brendler-Schwaab *et al.*, 2005). Interestingly, combining doxorubicin or etoposide with PI-103 significantly increased DNA damage compared with treatment with doxorubicin or etoposide alone (Figure 4a).

We then focused on doxorubicin, a drug which is used in treatment protocols for GBM (Hau *et al.*, 2004; Glas *et al.*, 2007), to investigate in more detail whether PI-103 affects DNA repair processes. To address this point, we monitored the resolution of DNA damage over a 24-h time span after pulse treatment with doxorubicin and/or PI-103 for 12 h. Importantly, the DNA damage caused by the combination of doxorubicin and PI-103 remained significantly elevated throughout this period compared with treatment with doxorubicin alone (Figure 4b).

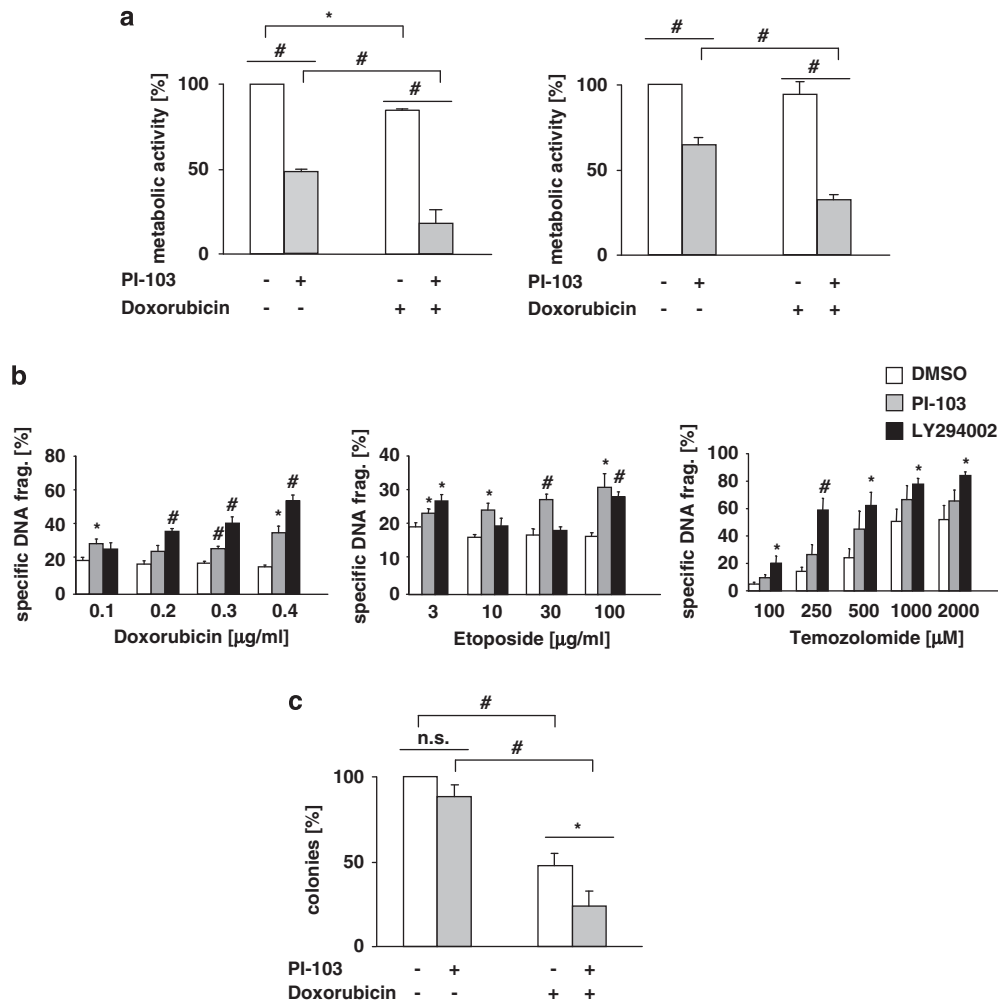
To further address whether it is the initial DNA damage or DNA repair processes that are affected by PI-103, we used an additional experimental approach, which allows for an earlier readout. As a surrogate



**Figure 2** PI-103 sensitizes glioblastoma cells for cytotoxic drug-induced apoptosis. (a–c) Cells were treated with indicated concentrations of doxorubicin for 48 h (a), with etoposide for 48 h (b) or with temozolomide for 144 h (c), and with dimethylsulfoxide (DMSO), PI-103 (U87MG: 0.6 µM; U138MG: 1.0 µM; U118MG: 0.06 µM) or 20 µM LY294002. Apoptosis was assayed by FACS analysis of the DNA fragmentation of propidium iodide-stained nuclei, and percentage of specific DNA fragmentation is shown. Mean + s.e.m. values of at least three independent experiments carried out in triplicate are shown. (d) U87MG cells were treated for 3 h with 0.3 µg/ml doxorubicin in the presence of DMSO or 0.6 µM PI-103, followed by a complete exchange of medium. On day 10, colony formation was assessed as described in the Materials and methods section, expressed here as a percentage of untreated cells under DMSO. Mean + s.d. values of three independent experiments are shown. Statistical analysis was carried out by two-sided Student's *t*-test, \**P* < 0.05; #*P* < 0.001; n.s., not significant.

marker of DNA damage, phosphorylation of histone H2A.X (referred to as  $\gamma$ H2A.X in its phosphorylated form) was analyzed, as the formation of  $\gamma$ H2A.X foci at the site of DNA damage is considered to be one of the earliest responses to DSBs and can be visualized by fluorescence microscopy (Fernandez-Capetillo *et al.*, 2003). To better distinguish between initial damage

and the onset of damage repair, the duration of drug treatment was reduced to 3 h. Thereafter, cells were released into a drug-free medium and a 24-h period was observed at intervals to monitor DNA repair. It is noteworthy that the initial damage after 3 h of exposure to doxorubicin was similar in the presence or absence of PI-103 (Figure 4c). Importantly, the resolution of



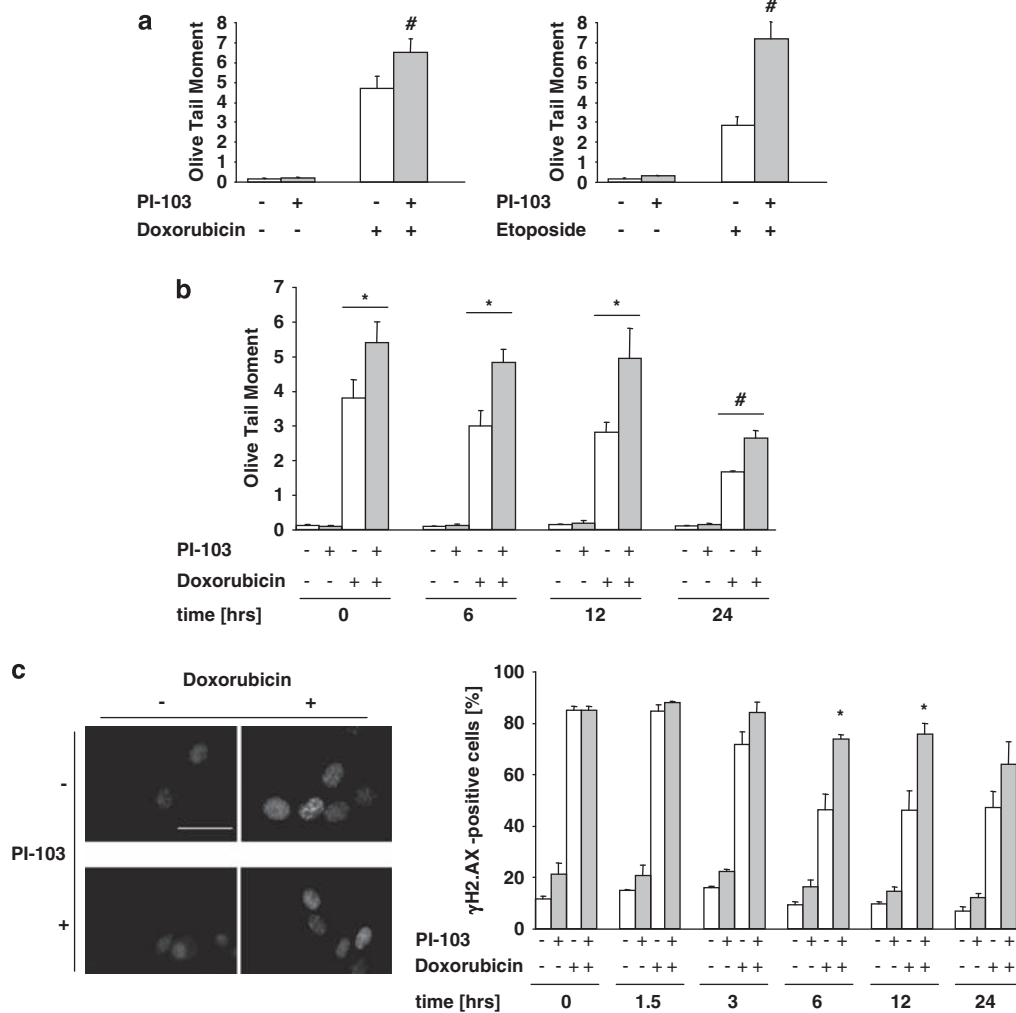
**Figure 3** PI-103 sensitizes glioblastoma (GBM) stem cells and primary cultured GBM cells for doxorubicin-induced apoptosis. (a) GBM tumor-initiating cells derived from two tumor specimens were treated for 72 h with 0.3 (left) or 0.1 (right) µg/ml doxorubicin in the presence of 0.6 µM PI-103 or dimethylsulfoxide (DMSO). Cell viability was determined by MTT assay and is expressed as a percentage of the metabolic activity that untreated cells exhibit under DMSO. Mean + s.e.m. values of three independent experiments carried out in triplicate are shown. (b) Primary cultured GBM cells were treated with indicated concentrations of doxorubicin or etoposide for 48 h or with temozolomide for 96 h in the presence of 0.6 µM PI-103 or DMSO. Apoptosis was assayed by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei and the percentage of specific DNA fragmentation is shown. Mean + s.e.m. values of at least three independent experiments carried out in triplicate are shown. (c) Primary cultured GBM cells were treated for 3 h with 0.3 µg/ml doxorubicin in the presence of 0.6 µM PI-103 or DMSO, followed by a complete exchange of medium. On day 7, colony formation was assessed as described in the Materials and methods section and is expressed as a percentage of untreated cells under DMSO. Mean + s.e.m. values of at least three independent experiments carried out in triplicate are shown. Statistical analysis was carried out by two-sided Student's *t*-test, \**P* < 0.05; #*P* < 0.001; n.s., not significant.

γH2A.X foci after drug removal was significantly delayed in cells that were co-treated with doxorubicin and PI-103, compared with treatment with doxorubicin alone (Figure 4b). This prolonged presence of γH2A.X foci in cells treated with PI-103 plus doxorubicin was unlikely simply because of cell cycle alterations, as these changes only became prominent after 48 h (compare Supplementary Figures 2b and c). Furthermore, we assessed γH2A.X foci formation in the presence of the broad-range caspase inhibitor, zVAD.fmk, to exclude that the persistence of γH2A.X foci was caused by increased caspase-mediated apoptotic DNA fragmentation. It is noteworthy that inhibition of caspases did not prevent the formation of γH2A.X foci (Supplementary

Figure 3a), indicating that γH2A.X foci formation occurs independent of caspase-mediated apoptotic events. These data taken together strongly argue for a model in which PI-103 sensitizes GBM cells for apoptosis by inhibiting DNA repair processes.

*The effect of PI3K inhibition and blockage of DNA-PK-mediated DNA repair are indistinguishable in sensitizing GBM cells for apoptosis*

PI3K may modulate DNA damage response through at least two different routes, that is, DNA-PK-mediated repair (Jackson, 2002) and mTOR-induced signaling (Proud, 2004). As PI-103 has been reported to inhibit

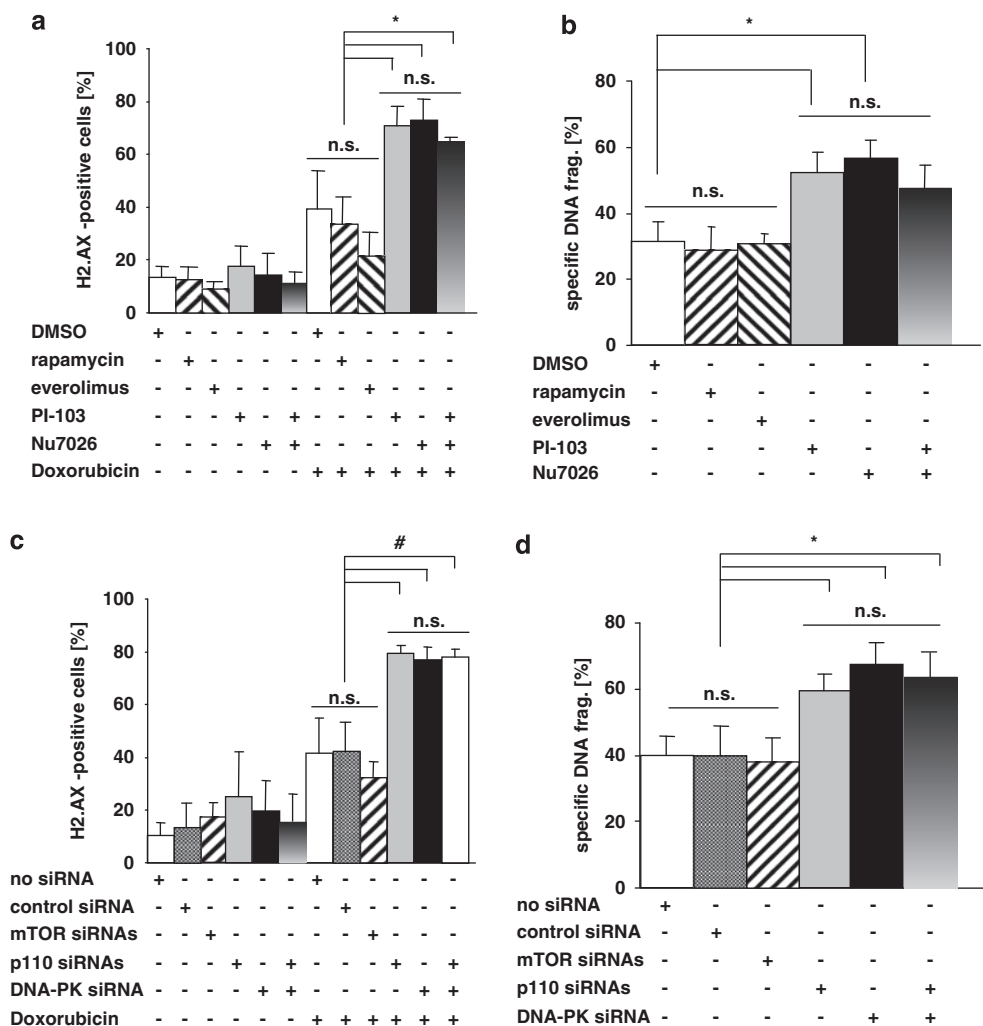


**Figure 4** PI-103 prolongs doxorubicin-induced DNA damage and retards clearance of  $\gamma$ H2A.X foci. (a) U87MG cells were treated for 12 h with 0.3  $\mu$ g/ml doxorubicin (left) or with 30  $\mu$ g/ml etoposide (right) in the presence of 0.6  $\mu$ M PI-103 or dimethylsulfoxide (DMSO). DNA damage was assayed by comet assay and is displayed as Olive Tail Moment. (b) U87MG cells were treated for 12 h with 0.3  $\mu$ g/ml doxorubicin in the presence of 0.6  $\mu$ M PI-103 or DMSO, followed by a complete exchange of medium. After the indicated time points, DNA damage was assayed by comet assay and is displayed as Olive Tail Moment, whereby the 0 h time point corresponds to 12 h drug treatment. (c) U87MG cells were treated for 3 h with 0.3  $\mu$ g/ml doxorubicin in the presence of 0.6  $\mu$ M PI-103 or DMSO, followed by a complete exchange of medium. After the indicated time points, DNA damage was assayed by scoring  $\gamma$ H2A.X foci-positive cells. Examples of  $\gamma$ H2A.X foci-positive cells are shown on the left (scale bar: 50  $\mu$ m) and the percentage of  $\gamma$ H2A.X foci-positive cells is shown on the right (whereby the 0 h time point corresponds to 3 h drug treatment). In panels a and b, the median + s.d. values of three independent experiments are shown, whereas in panel (c), the mean + s.d. values of three independent experiments are depicted. Statistical analysis was carried out using the Mann-Whitney *U*-test in a and b and two-sided Student's *t*-test in C., \**P* < 0.05; #*P* < 0.001.

DNA-PK and both mTORC1 and mTORC2 complexes, besides class I PI3K (Raynaud *et al.*, 2007), we wanted to critically analyze their individual contributions to PI-103-mediated chemosensitization. To address this point, we used both, a pharmacological and a genetic approach.

In the first approach, inhibition of the catalytic subunit of DNA-PK by the pharmacological inhibitor Nu7026 (Veuger *et al.*, 2003) led to a similar increase in  $\gamma$ H2A.X foci formation when coadministered with doxorubicin, as PI-103 did (Figure 5a). Importantly, using a combination of both pharmacological inhibitors, that is, PI-103 and Nu7026, together with doxorubicin, did not further increase  $\gamma$ H2A.X foci numbers com-

pared with the use of doxorubicin and a single inhibitor (Figure 5a). Similarly, treatment with doxorubicin in combination with either PI-103 or Nu7026 significantly increased apoptosis compared with treatment with doxorubicin alone, although combining both pharmacological inhibitors did not lead to a further augmentation of cell death (Figure 5b). These results suggest that both PI3K and DNA-PK function in the same, linear signaling cascade. In contrast, two mTOR inhibitors, rapamycin and everolimus, had no effect on either DNA repair or apoptosis induction on treatment with doxorubicin (Figures 5a and b). Although these mTOR inhibitors primarily target mTORC1, they have also been reported to reduce mTORC2 levels on prolonged



**Figure 5** Comparison of pharmacological and genetic inhibition of PI3K, DNA-PK and mTOR. U87MG cells were treated with 100 nM rapamycin, 10 nM everolimus, 0.6  $\mu$ M PI-103, 10  $\mu$ M Nu7026 or dimethylsulfoxide (DMSO), and with either 0.3  $\mu$ g/ml doxorubicin for 3 h followed by a complete exchange of medium and determination of DNA damage by  $\gamma$ H2A.X foci formation after 12 h (a) or 0.2  $\mu$ g/ml doxorubicin for 48 h followed by determination of apoptosis by FACS analysis of propidium iodide-stained nuclei (b). (c and d) Genetic inhibition of PI3K, DNA-PK and mTOR. U87MG cells were treated with control siRNA, or siRNA against PI3K, DNA-PK or mTOR and with either 0.3  $\mu$ g/ml doxorubicin for 3 h followed by a complete exchange of medium and determination of DNA damage by  $\gamma$ H2A.X foci formation after 12 h (c) or 0.2  $\mu$ g/ml doxorubicin for 48 h followed by determination of apoptosis by FACS analysis of propidium iodide-stained nuclei (d). Mean  $\pm$  s.d. values of three independent experiments carried out in triplicate (a and c) or mean  $\pm$  s.e.m. values of three independent experiments carried out in triplicate (b and d) are shown. Statistical analysis was carried out by two-sided Student's *t*-test, \* $P$  < 0.05; # $P$  < 0.001; n.s., not significant.

exposure (Sarbassov *et al.*, 2006; Guertin and Sabatini, 2007).

In the second, genetic approach, expression of key targets of PI-103 was individually knocked down by RNA interference (the effectiveness and specificity of which are shown in Supplementary Figure 3b). To this end, PI3K was targeted by a simultaneous knockdown of the p110 $\alpha$  and p110 $\beta$  forms of the p110 subunit, DNA-PK by knockdown of the DNA-PK catalytic subunit and mTOR by knockdown of FRAP1/mTOR, the essential catalytic protein of both mTORC1 and mTORC2 complexes. Intriguingly, a selective knockdown of either PI3K or DNA-PK significantly increased both,  $\gamma$ H2A.X foci numbers (Figure 5c) and doxorubicin-induced apoptosis (Figure 5d). Crucially, the com-

bined knockdown of PI3K and DNA-PK did not further increase  $\gamma$ H2A.X foci formation or apoptosis, when compared with the knockdown of individual proteins (Figures 5c and d). In contrast, knockdown of mTOR did not significantly alter doxorubicin-induced  $\gamma$ H2A.X foci formation or apoptosis (Figures 5c and d), which is in line with our findings for pharmacological mTOR inhibitors (Figures 5a and b). To further verify that reduced DNA repair is indeed specific for the inhibition of PI3K-mediated signaling, we also investigated Rad51, a protein involved in PI3K-independent homologous recombination (Jackson, 2002), a process that is also activated after doxorubicin treatment (Koehn *et al.*, 2007). Rad51-mediated damage response is indeed unaltered by both PI-103 and Nu7026

treatment (Supplementary Figure 3d), further strengthening the direct link between PI3K inhibition and reduction in NHEJ.

Together, these findings strongly suggest that PI-103 sensitizes GBM cells for doxorubicin-induced apoptosis by inhibiting DNA-PK-mediated DNA repair.

## Discussion

In this study, we provide a compelling argument for further investigating the use of class I PI3K inhibitors, such as the pyridinylfuranopyrimidine inhibitor, PI-103, in combination with DNA-damaging drugs for GBM treatment. Although it has been shown earlier that PI-103 has an anti-proliferative effect on GBM cells as a single agent (Fan *et al.*, 2007) or in combination with the epidermal growth factor receptor inhibitor erlotinib (Fan *et al.*, 2006), and that it enhances tumor radiosensitivity (Prevo *et al.*, 2008), no study has so far addressed the potential interaction between chemotherapeutic agents and PI-103. This is of particular interest for GBM, for which the current standard of care is still considered to be a surgical resection of the tumor mass, a frequently unsuccessful and difficult procedure (Stupp *et al.*, 2006).

In this study, we show for the first time that PI-103 efficiently sensitizes GBM cells for chemotherapy-induced apoptosis, not only established cell lines but also GBM stem cells. These cells are considered to be highly resistant to therapy and their presence has been frequently cited as a likely cause for tumor relapse (Ishii *et al.*, 2008). Furthermore, this PI-103-mediated increase in chemosensitivity is most likely of clinical relevance, as we also observed increased apoptosis after a combination therapy of doxorubicin and PI-103 in primary GBM cells derived from patients.

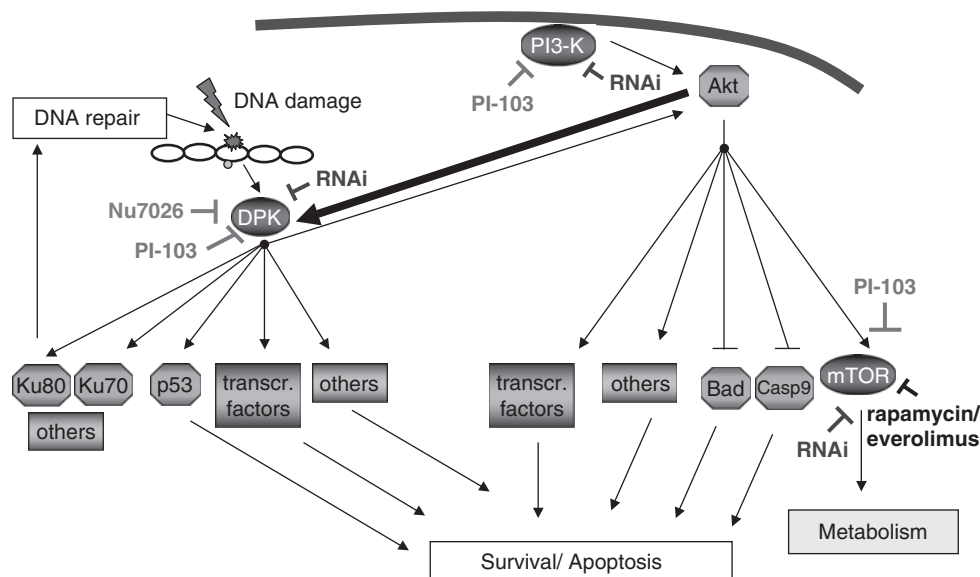
Investigating the molecular mechanisms of this chemosensitization, we found that inhibition of PI3K led to persistent DNA damage and inhibition of DNA repair after doxorubicin treatment. Although the relationship between inhibition of PI3K signaling and increased DNA damage has been reported in the context of tumor radiosensitivity, increased either by LY294002 (Kao *et al.*, 2007) or PI-103 (Prevo *et al.*, 2008), the underlying molecular mechanism has so far not been investigated. Previously, distinct routes by which PI3K-mediated signaling could influence cellular DNA damage responses were suggested, such as interaction with DNA-PK-dependent DNA repair (Jackson, 2002). In addition, it has been shown that mTOR-induced signaling can influence cellular responses to DNA damage (Proud, 2004). Interestingly, both proteins, the catalytic subunit of DNA-PK and mTOR, are also inhibited by PI-103 (Raynaud *et al.*, 2007). Thus, the question arises which of these potential targets of PI-103 are crucial for mediating chemosensitization on treatment with DNA-damaging drugs. Our pharmacological and genetic approach to individually inhibit these molecules suggests a linear relationship between PI3K

and DNA-PK, as blocking either PI3K or DNA-PK resulted in a similar delay of DNA repair accompanied by increased apoptosis, whereas a simultaneous inhibition of both molecules did not lead to any additive or synergistic effects.

The interaction between the NHEJ DNA repair pathway, of which DNA-PK is an integral part, and the PI3K/Akt signaling cascade is highly complex and still not fully understood (Lees-Miller, 2008). In non-transformed cells, DNA-PK is usually but not always (for example, Yavuzer *et al.*, 1998) believed to be directly activated on a DSB (Pawelczak and Turchi, 2008), thereafter initiating the repair machinery while concurrently activating survival pathways, such as PI3K/Akt-mediated signaling (Bozulic *et al.*, 2008), possibly by direct phosphorylation (Feng *et al.*, 2004). In tumor cells, however, which are usually more dependent on PI3K-mediated survival signaling and frequently express stimuli-independent activity of this signaling cascade (Yuan and Cantley, 2008), the relationship between PI3K and DNA-PK can be altered. Here, Akt has been reported to be an activator of DNA-PK, responsible for its phosphorylation (Toulany *et al.*, 2006, 2008). These findings are consistent with our own data, which show that in doxorubicin-treated GBM cells, Akt is not downstream of DNA-PK, as inhibition of the latter does not affect phosphorylation of the former (Supplementary Figure 3d).

Although it has been previously suggested that mTOR may mediate cellular responses to DNA damage-induced apoptosis (Proud, 2004), our results indicate that, in contrast to PI3K and DNA-PK, it does not seem to have a crucial role in mediating DNA repair or apoptosis resistance after doxorubicin treatment in GBM cells. However, it may remain an interesting target for therapeutic approaches in GBM (Kreisl *et al.*, 2009). Indeed, compounds inhibiting multiple targets, such as PI3K, DNA-PK and mTOR, seem to be a promising strategy, as blocking a single target has been shown to increase the selective pressure for the development of mutational resistance to that substance. Therefore, targeting a range of overlapping signaling pathways, which are all dependent on a protein family that is aberrantly activated in tumor cells, is a much more stable approach, which still avoids the pitfalls of classical broad-spectrum inhibition.

Our findings, as summarized in Figure 6, strongly suggest that inhibition of DNA-PK-mediated DNA repair is involved in PI-103-mediated sensitization when treating GBM cells with the DNA-damaging drug, doxorubicin. This might also explain in part the reduced sensitization effect of PI-103 when combined with temozolomide, which is an alkylating agent (Tisdale, 1985) and damages DNA by generating O<sup>6</sup>-alkylguanine, which is repaired primarily independent of DNA-PK (Christmann *et al.*, 2003). Indeed, inhibition of PI3K-mediated signaling also enhances apoptosis sensitivity in GBM cells in an NHEJ-independent manner, as we have previously shown for agents that do not primarily cause DNA damage, that is, death receptor ligands such as TRAIL and anti-CD95 agonistic



**Figure 6** A working model of the interactions between PI3K/Akt/mTOR signaling and DNA repair. In glioblastoma cells, the nonhomologous end-joining DNA repair pathway gets activated on induction of DNA damage (here, depicted as a red lightning bolt), whereas the PI3K/Akt pathway is active. This leads to the activation of DNA-PK. Both DNA-PK and PI3K-mediated signaling lead to the (in)activation of numerous downstream targets with the net effect of initiating DNA repair, sustaining cell survival and inhibiting apoptosis. In addition, PI3K-mediated signaling through mTOR controls metabolic processes. Inhibition of the key components (blue ovals) of this network by pharmacological inhibitors (PI-103, Nu7026) or RNA interference (RNAi) indicates a linear relationship between PI3K and DNA-PK in the regulation of DNA repair and apoptosis. A full colour version of this figure is available at the *Oncogene* journal online.

antibody (Opel *et al.*, 2008). Intriguingly, in line with recent observations regarding the pharmacokinetics of inhibitor-induced cancer cell death (Shah *et al.*, 2008), we also found that a brief, but potent inhibition of PI3K administered concurrently with a DNA-damaging drug is sufficient to commit GBM cells to apoptosis and to suppress clonogenic growth.

We therefore conclude that pharmacological inhibitors of class I PI3K, such as PI-103, are promising candidates for further combination protocols with chemotherapeutic drugs such as doxorubicin, which is currently being evaluated for treatment of GBM (Hau *et al.*, 2004; Glas *et al.*, 2007). By inhibiting DNA-PK-mediated DNA repair, PI-103 chemosensitizes GBM cell lines, primary material of patients and also glioma stem cells for apoptosis. Thus, our findings show that inhibition of PI3K-mediated signaling is a promising therapeutic approach to overcome resistance of GBM. This is of particular interest as a recent study by Chen *et al.* (2008) shows a similar sensitizing effect in GBM after radiation treatment combined with PI-103 (Chen *et al.*, 2008). Although the authors did not investigate the underlying mechanisms, similar pathways as those described in this study may be involved.

## Materials and methods

### Cells culture

GBM cell lines (obtained from ATCC, Manassas, VA, USA), primary cultured patient material (as characterized in Opel *et al.*, 2008) and GBM-initiating cells (as characterized in Eramo *et al.*, 2006) were cultured as described (Opel *et al.*,

2008; Eramo *et al.*, 2006, respectively). Temozolomide was obtained from Schering-Plough (Kenilworth, NJ, USA), PI-103 from Alexis Biochemicals (Lörrach, Germany) and everolimus was kindly provided by Novartis Institute for BioMedical Research (Oncology Basel, Novartis Pharma AG, Basel, Switzerland). All chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany), unless indicated otherwise.

### Protein immunoblotting

Western blot analysis was carried out as previously described (Opel *et al.*, 2008), using the following antibodies: mouse monoclonal Akt (BD Biosciences, Heidelberg, Germany); rabbit polyclonal phospho-Akt (Ser473), rabbit phospho-S6 ribosomal protein (Ser235/236), rabbit anti-S6 ribosomal protein (Cell Signaling, Beverly, MA, USA); mouse monoclonal  $\beta$ -actin (Sigma-Aldrich), followed by anti-mouse or anti-rabbit immunoglobulin G-horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany).

### Determination of apoptosis, viability and colony formation

The readout for apoptosis was DNA fragmentation and was assessed by fluorescence-activated cell-sorting (FACScan, Becton Dickinson, Heidelberg, Germany) analysis of DNA fragmentation of propidium iodide-stained nuclei as previously described (Fulda *et al.*, 1997). Specific DNA fragmentation was calculated as follows:  $100 \times (\text{experimental DNA fragmentation (\%)} - \text{spontaneous DNA fragmentation (\%)}) / (100\% - \text{spontaneous DNA fragmentation (\%)})$ . The readout for cell viability was metabolic activity and was assessed by MTT assay according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). For colony assay,  $2 \times 10^3$  U87MG or  $8 \times 10^3$  primary cultured cells were seeded in 6-well plates and treated for 3 h with 0.3  $\mu\text{g/ml}$  doxorubicin in the

presence of PI-103 or dimethylsulfoxide. The media were replaced by a drug-free medium and cells were allowed to grow for 10 days (U87MG cells) or for 7 days (primary cultured cells). At the end of the incubation period, cells were washed twice in phosphate-buffered saline (PBS), followed by a 15 min fixation with 3.7% formaldehyde (in PBS) and a 10 min staining in a Giemsa (Merck KGaA, Darmstadt, Germany) solution (1:10 in water). Clusters of at least 10 cells were counted as colonies and values were normalized for untreated cells under dimethylsulfoxide.

#### Single-cell gel electrophoresis (comet) assay

DNA damage was assayed by alkaline comet assay. A total of  $2 \times 10^5$  U87MG cells were seeded in 6-cm dishes and allowed to settle overnight. After drug exposure, the cells were washed with PBS, collected by centrifugation and resuspended in PBS. Aliquots of 10  $\mu$ l were suspended in 120  $\mu$ l low melting point agarose (0.5%) (Invitrogen, Karlsruhe, Germany) and spread onto microscope slides precoated with a thin layer of 1.5% agarose (Roth, Karlsruhe, Germany). The cells were exposed to lysis buffer (2.5 mM NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris) at 4 °C overnight. Thereafter, alkaline denaturation was carried out in a prechilled electrophoresis buffer (300 mM NaOH, 1 mM Na<sub>2</sub>EDTA) for 25 min, followed by alkaline electrophoresis for 25 min at a pH > 13 (4 °C). The slides were then neutralized (0.4 M Tris), desiccated (absolute alcohol, 5 min) and stained with ethidium bromide. A total of 50 randomly selected cells were measured by image analysis (Kinetic Imaging Komet 5.0 Software, Andor Technology Ltd, Berlin, Germany) using an Olympus AX70 'Provis' microscope (Hamburg, Germany). DNA damage is expressed as Olive Tail Moment.

#### $\gamma$ H2A.X foci formation

The U87MG cells were seeded at a density of  $0.5 \times 10^5$  in 8-well chamber slides and stained with anti-phospho-Histone H2A.X (Millipore, Schwalbach/Ts., Germany) and anti-mouse fluorescein-conjugated F(ab')<sub>2</sub> IgG (Millipore) as previously

described (Giagkousiklidis *et al.*, 2005). Cells were analyzed under a fluorescence microscope. Nuclei containing at least 10 fluorescence foci were considered positive, and for each experimental condition, at least 200 cells were counted.

#### RNA interference

Cells were transfected with siRNA using TransMessenger transfection reagent (Qiagen, Hilden, Germany) as previously described (Opel *et al.*, 2008), using the following Stealth RNAi constructs from Invitrogen: PIK3CA (PIK3CAHSS10800 6) and PIK3CB (PIK3CBHSS10800 7) for the p110 $\alpha$  and  $\beta$  subunits of PI3K; PRKDC (PRKCDHSS10852 7) for the catalytic subunit of DNA-PK; FRAP1 (FRAP1HSS103825 5–7) for mTOR; and Stealth Negative Universal Control Medium RNAi (12935–300) for negative control.

#### Statistical analysis

Statistical significance was assessed by Student's *t*-test or Mann–Whitney *U*-test, where appropriate, using Winstat (R. Fitch Software, Bad Krozingen, Germany) or SPSS (SPSS GmbH Software, Munich, Germany) software.

#### Conflict of interest

The authors declare no conflict of interest.

#### Acknowledgements

We thank H Lane (Novartis Institute for BioMedical Research, Oncology Basel, Novartis Pharma AG, Basel, Switzerland) for providing everolimus, R Pallini and R De Maria for glioblastoma-initiating cells and S Piater for expert technical assistance. This work was supported by grants from Deutsche Forschungsgemeinschaft, Deutsche Krebshilfe, European Community (ApopTrain, APO-SYS), Novartis Stiftung für therapeutische Forschung and IAP6/18 (to SF), and the University of Ulm junior research grant (to JAK).

#### References

- Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB *et al.* (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**: 756–760.
- Bozulic L, Surucu B, Hynx D, Hemmings BA. (2008). PKB/alpha/Akt1 acts downstream of DNA-PK in the DNA double-strand break response and promotes survival. *Mol Cell* **30**: 203–213.
- Brendler-Schwaab S, Hartmann A, Pfuhler S, Speit G. (2005). The *in vivo* comet assay: use and status in genotoxicity testing. *Mutagenesis* **20**: 245–254.
- Cancer Genome Atlas Research Network (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* **455**: 1061–1068.
- Cavaliere R, Wen PY, Schiff D. (2007). Novel therapies for malignant gliomas. *Neurol Clin* **25**: 1141–1171, x.
- Chakravarti A, Zhai G, Suzuki Y, Sarkesh S, Black PM, Muzikansky A *et al.* (2004). The prognostic significance of phosphatidylinositol 3-kinase pathway activation in human gliomas. *J Clin Oncol* **22**: 1926–1933.
- Chen JS, Zhou LJ, Entin-Meer M, Yang X, Donker M, Knight ZA *et al.* (2008). Characterization of structurally distinct, isoform-selective phosphoinositide 3'-kinase inhibitors in combination with radiation in the treatment of glioblastoma. *Mol Cancer Ther* **7**: 841–850.
- Christmann M, Tomicic MT, Roos WP, Kaina B. (2003). Mechanisms of human DNA repair: an update. *Toxicology* **193**: 3–34.
- Colman H, Aldape K. (2008). Molecular predictors in glioblastoma: toward personalized therapy. *Arch Neurol* **65**: 877–883.
- DeAngelis LM. (2001). Brain tumors. *N Engl J Med* **344**: 114–123.
- Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G *et al.* (2006). Chemotherapy resistance of glioblastoma stem cells. *Cell Death Differ* **13**: 1238–1241.
- Fan QW, Cheng CK, Nicolaidis TP, Hackett CS, Knight ZA, Shokat KM *et al.* (2007). A dual phosphoinositide-3-kinase alpha/mTOR inhibitor cooperates with blockade of epidermal growth factor receptor in PTEN-mutant glioma. *Cancer Res* **67**: 7960–7965.
- Fan QW, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D *et al.* (2006). A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* **9**: 341–349.
- Feng J, Park J, Cron P, Hess D, Hemmings BA. (2004). Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. *J Biol Chem* **279**: 41189–41196.
- Fernandez-Capetillo O, Celeste A, Nussenzweig A. (2003). Focusing on foci: H2AX and the recruitment of DNA-damage response factors. *Cell Cycle* **2**: 426–427.
- Fruman DA, Meyers RE, Cantley LC. (1998). Phosphoinositide kinases. *Annu Rev Biochem* **67**: 481–507.

- Fulda S, Sieverts H, Friesen C, Herr I, Debatin KM. (1997). The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. *Cancer Res* **57**: 3823–3829.
- Giagkousiklidis S, Vogler M, Westhoff MA, Kasperczyk H, Debatin KM, Fulda S. (2005). Sensitization for gamma-irradiation-induced apoptosis by second mitochondria-derived activator of caspase. *Cancer Res* **65**: 10502–10513.
- Glas M, Koch H, Hirschmann B, Jauch T, Steinbrecher A, Herrlinger U et al. (2007). Pegylated liposomal doxorubicin in recurrent malignant glioma: analysis of a case series. *Oncology* **72**: 302–307.
- Guertin DA, Sabatini DM. (2007). Defining the role of mTOR in cancer. *Cancer Cell* **12**: 9–22.
- Hau P, Fabel K, Baumgart U, Rummele P, Grauer O, Bock A et al. (2004). Pegylated liposomal doxorubicin-efficacy in patients with recurrent high-grade glioma. *Cancer* **100**: 1199–1207.
- Ihle NT, Powis G. (2009). Take your PIK: phosphatidylinositol 3-kinase inhibitors race through the clinic and toward cancer therapy. *Mol Cancer Ther* **8**: 1–9.
- Ishii H, Iwatsuki M, Ieta K, Ohta D, Haraguchi N, Mimori K et al. (2008). Cancer stem cells and chemoradiation resistance. *Cancer Sci* **99**: 1871–1877.
- Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC et al. (1999). Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. *Brain Pathol* **9**: 469–479.
- Jackson SP. (2002). Sensing and repairing DNA double-strand breaks. *Carcinogenesis* **23**: 687–696.
- Jiang BH, Liu LZ. (2008). PI3K/PTEN signaling in tumorigenesis and angiogenesis. *Biochim Biophys Acta* **1784**: 150–158.
- Kao GD, Jiang Z, Fernandes AM, Gupta AK, Maity A. (2007). Inhibition of phosphatidylinositol-3-OH kinase/Akt signaling impairs DNA repair in glioblastoma cells following ionizing radiation. *J Biol Chem* **282**: 21206–21212.
- Koehn H, Magan N, Isaacs RJ, Stowell KM. (2007). Differential regulation of DNA damage repair protein Rad51 in human tumour cell lines exposed to doxorubicin. *Anticancer Drugs* **18**: 419–425.
- Kreisl TN, Lassman AB, Mischel PS, Rosen N, Scher HI, Teruya-Feldstein J et al. (2009). A pilot study of everolimus and gefitinib in the treatment of recurrent glioblastoma (GBM). *J Neurooncol* **92**: 99–105.
- Lees-Miller SP. (2008). PIKK-ing a new partner: a new role for PKB in the DNA damage response. *Cancer Cell* **13**: 379–380.
- Maira SM, Stauffer F, Schnell C, Garcia-Echeverria C. (2009). PI3K inhibitors for cancer treatment: where do we stand? *Biochem Soc Trans* **37**: 265–272.
- Maira SM, Voliva C, Garcia-Echeverria C. (2008). Class IA phosphatidylinositol 3-kinase: from their biologic implication in human cancers to drug discovery. *Expert Opin Ther Targets* **12**: 223–238.
- Newton HB. (2008). Glioblastoma multiforme. *Curr Treat Options Neurol* **10**: 285–294.
- Nitiss JL. (2002). DNA topoisomerases in cancer chemotherapy: using enzymes to generate selective DNA damage. *Curr Opin Investig Drugs* **3**: 1512–1516.
- Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL et al. (2004). Genetic pathways to glioblastoma: a population-based study. *Cancer Res* **64**: 6892–6899.
- Opel D, Poremba C, Simon T, Debatin KM, Fulda S. (2007). Activation of Akt predicts poor outcome in neuroblastoma. *Cancer Res* **67**: 735–745.
- Opel D, Westhoff MA, Bender A, Braun V, Debatin KM, Fulda S. (2008). Phosphatidylinositol 3-kinase inhibition broadly sensitizes glioblastoma cells to death receptor- and drug-induced apoptosis. *Cancer Res* **68**: 6271–6280.
- Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P et al. (2008). An integrated genomic analysis of human glioblastoma multiforme. *Science* **321**: 1807–1812.
- Pawelczak KS, Turchi JJ. (2008). A mechanism for DNA-PK activation requiring unique contributions from each strand of a DNA terminus and implications for microhomology-mediated nonhomologous DNA end joining. *Nucleic Acids Res* **36**: 4022–4031.
- Prevo R, Deutsch E, Sampson O, Diplexcito J, Cengel K, Harper J et al. (2008). Class I PI3 kinase inhibition by the pyridinylfuranopyrimidine inhibitor PI-103 enhances tumor radiosensitivity. *Cancer Res* **68**: 5915–5923.
- Proud CG. (2004). The multifaceted role of mTOR in cellular stress responses. *DNA Repair (Amst)* **3**: 927–934.
- Raynaud FI, Eccles S, Clarke PA, Hayes A, Nutley B, Alix S et al. (2007). Pharmacologic characterization of a potent inhibitor of class I phosphatidylinositol 3-kinases. *Cancer Res* **67**: 5840–5850.
- Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF et al. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* **22**: 159–168.
- Shah NP, Kasap C, Weier C, Balbas M, Nicoll JM, Bleickardt E et al. (2008). Transient potent BCR-ABL inhibition is sufficient to commit chronic myeloid leukemia cells irreversibly to apoptosis. *Cancer Cell* **14**: 485–493.
- Stupp R, Hegi ME, van den Bent MJ, Mason WP, Weller M, Mirimanoff RO et al. (2006). Changing paradigms—an update on the multidisciplinary management of malignant glioma. *Oncologist* **11**: 165–180.
- Tisdale MJ. (1985). Antitumour imidazotetrazines—XI: effect of 8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one [CCRG 81045; M and B 39831 NSC 362856] on poly(ADP-ribose) metabolism. *Br J Cancer* **52**: 789–792.
- Toulany M, Kasten-Pisula U, Wang S, Chen J, Dittmann K et al. (2006). Blockage of epidermal growth factor receptor-phosphatidylinositol 3-kinase-AKT signaling increases radiosensitivity of K-RAS mutated human tumor cells *in vitro* by affecting DNA repair. *Clin Cancer Res* **12**: 4119–4126.
- Toulany M, Kehlbach R, Florczak U, Sak A, Wang S, Chen J et al. (2008). Targeting of AKT1 enhances radiation toxicity of human tumor cells by inhibiting DNA-PKcs-dependent DNA double-strand break repair. *Mol Cancer Ther* **7**: 1772–1781.
- Veuger SJ, Curtin NJ, Richardson CJ, Smith GC, Durkacz BW. (2003). Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1. *Cancer Res* **63**: 6008–6015.
- Yaneva M, Li H, Marple T, Hasty P. (2005). Non-homologous end joining, but not homologous recombination, enables survival for cells exposed to a histone deacetylase inhibitor. *Nucleic Acids Res* **33**: 5320–5330.
- Yap TA, Garrett MD, Walton MI, Raynaud F, de Bono JS, Workman P. (2008). Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. *Curr Opin Pharmacol* **8**: 393–412.
- Yavuzer U, Smith GC, Bliss T, Werner D, Jackson SP. (1998). DNA end-independent activation of DNA-PK mediated via association with the DNA-binding protein C1D. *Genes Dev* **12**: 2188–2199.
- Yuan TL, Cantley LC. (2008). PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**: 5497–5510.

Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)