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Enzastaurin induces H2AX phosphorylation to regulate apoptosis via MAPK signalling in malignant glioma cells

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ABSTRACT

Enzastaurin is an acyclic bisindolylmaleimide derived from staurosporine that acts as an ATP competitor, and interferes with the activity of protein kinase C (PKC) isoforms. Our previous studies have shown that clinically achievable concentrations of this agent induce apoptosis in many glioma cell lines. Our goal in this study was to expand on the previous results and to determine the signalling mechanisms responsible for enzastaurin-induced inhibition of cell growth and induction of apoptosis. To address these issues, cell cycle progression following enzastaurin treatment was analysed by fluorescence-activated cell sorting (FACS) in parallel with analyses of growth and apoptosis signalling pathways. Enzastaurin treatment activated H2AX and Chk2 phosphorylation, and enhanced phosphorylation of mitogen-activated protein kinase (MAPK) family kinases. Inhibition of MAP kinases by chemical inhibitors reduced H2AX and Chk2 phosphorylation and decreased apoptosis induced by enzastaurin. These data call attention to a novel signalling pathway (MAPK/H2AX) to regulate apoptosis in malignant glioma cells.

1. Introduction

Malignant gliomas are aggressive tumours that generally prove refractory to treatment with surgery, irradiation and conventional chemotherapy. These tumours characteristically harbour a variety of genetic alterations that facilitate cell proliferation and survival.[1,2] Recent studies from our laboratory[3–5] and others[6] have noted that the aberrant proliferation of these tumours may in part reflect the effects of dysregulated growth factor-receptor-mediated signalling on downstream targets, such as protein kinase C (PKC), leading to constitutive activation of growth-promoting isoforms. Accordingly, significant interest has been directed at inhibiting PKC and other downstream kinase targets as a way of interfering with glioma cell growth.[4,5,7,8]

Enzastaurin is a bisindolylmaleimide derivative that effectively inhibits several PKC isoforms, and is currently undergoing clinical trials in several types of cancers.[9,10] In previous studies with this agent, we[3] observed cytotoxic activity against malignant glioma cell lines, and partial abrogation of cell proliferation with clinically achievable drug concentrations. In this study we sought to determine the signalling mechanisms responsible for enzastaurin-induced inhibition of cell growth and induction of apoptosis in glioma cell lines. We therefore examined the effect of enzastaurin on the activation of mitogen-activated protein kinase (MAPK) family members, in parallel with the analyses of activation of apoptotic pathway signalling and cell cycle progression. MAPK family members play an evolutionarily conserved role in mediating and amplifying growth factor–mediated and mito-

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genic signals from the cytoplasm to the nucleus.[11] Three major MAPK pathways have been described; the extracellular signal-related kinase (ERK), the c-Jun NH2-terminal kinase (JNK) and the p38 MAPK pathways. In addition to their roles in regulating normal cell growth, all three classes are also known to be activated in response to stress stimuli such as ionising radiation (IR) and UV light. The MAPKs work in concert to balance cell death with growth and survival. Deregulation of the MAPK pathways is associated with genomic instability and cancer.

Another highly conserved cellular process is the repair of DNA double-strand breaks (DSBs). In response to double-strand breaks, the cell triggers checkpoints that halt the cell cycle while a decision is made regarding repair and survival or death.[12] DNA damage activates a cascade of protein kinases that relay the signal to downstream effectors to halt the cell cycle and that facilitate repair of the damage.[13] One of the earliest events in the normal cellular response to DSBs is the phosphorylation by ataxia telangiectasia mutated protein (ATM) of a histone H2A variant, H2AX, at sites of DNA damage.[14–16] H2AX is rapidly phosphorylated (within minutes) at an evolutionarily conserved residue, Ser139, when DSBs are induced in mammalian cells, resulting in discrete phosphorylated H2AX (γ H2AX) foci at or near the DNA damage sites, and constituting a marker to correlate DNA damage with cell cycle phase or induction of apoptosis.[14,15] Moreover, the intensity of H2AX immunofluorescence (IF) measured by cytometry has been reported to correlate with the frequency of DSBs induced by X-ray radiation or by DNA damaging anti-tumour drugs.

Several members of the MAPK family have been linked to the DNA-damage response and ATM-mediated signalling events. For example, low levels of DNA damage can trigger prosurvival signals mediated by ERK1/2 phosphorylation,[17] p38 MAPK triggers G2-M arrest in response to ionising irradiation in an ATM-dependent manner,[18] and JNK activation has been shown to promote base excision repair of cisplatin-induced DNA lesions.[19]

Herein, we report that enzastaurin-promoted apoptosis in malignant human glioma cells is associated with significant activation of MAPK family members and H2AX phosphorylation. Rather than being a compensatory mechanism for cellular resistance to enzastaurin, MAPK family activation appeared to play a role in the apoptotic signalling cascade induced by this agent. Accordingly, inhibition of individual MAPK family members completely abolished enzastaurin-induced H2AX phosphorylation and rescued glioma cells from apoptosis.

2. Materials and methods

2.1. Reagents and cell culture

Enzastaurin was obtained from Eli Lilly (Indianapolis, IN). Stock aliquots (10 mM) were stored at −20 °C. U0126, SB203580 and SP600125 were purchased from Calbiochem. The established malignant glioma cell lines U87, T98G and A172 were obtained from the American Type Culture Collection (Manassas, VA). LNZ428 and LNZ308 were generously provided by Dr. Nicolas de Tribolet. U87 and T98G were cultured in growth medium composed of minimum essential medium; A172, LNZ428 and LNZ308 were cultured in α-minimal essential medium. All growth media contained 10% foetal calf serum, non-essential amino acids, 100 IU/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin (Invitrogen, Bethesda, MD).

2.2. Cell cycle analysis

The effect of enzastaurin on cell cycle distribution was determined by flow cytometric analysis of the DNA content of T98G cell nuclei following staining with propidium iodide. Briefly, cells grown exponentially to 40–50% confluency were exposed to enzastaurin or DMSO for 24 h, harvested, washed briefly in ice-cold PBS and fixed in 70% ethanol. DNA was stained by incubating the cells in PBS containing propidium iodide (50 μg/ml) and RNase A (1 mg/ml) for 60 min at room temperature, and fluorescence was measured and analysed using a Becton Dickinson FACScan with Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

2.3. Cell proliferation assay

Cells (1×10^3/well) were plated in 96-well microtiter plates (Costar, Cambridge, MA) in 100 μl of growth medium, and after overnight attachment were exposed to inhibitors for 3 days. Control cells received vehicle alone (DMSO). After the treatment interval, cells were washed in inhibitor-free medium, and the number of viable cells was determined using a colorimetric cell proliferation assay (CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega), which measures the bioreduction of the tetrazolium compound 3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2*H* tetrazolium (MTS) by dehydrogenase enzymes of metabolically active cells into a soluble formazan product, in the presence of the electron coupling reagent phenazine methosulphate.[20] All studies were conducted in triplicate and repeated at least three times independently. To perform the assay, 20 μl of MTS/phenazine methosulphate solution was added to each well, and after 1 h of incubation at 37 °C in a humidified 5% CO_2 atmosphere, absorbance was measured at 490 nm in a microplate reader. Triplicate wells with predetermined cell numbers were subjected to the above-mentioned assay in parallel with the test samples to normalise the absorbance readings.

2.4. Clonogenic growth assay

A direct assessment of the effect of different inhibitor concentrations on cell viability was performed using a clonogenic assay. For these studies, 200 cells were plated in 6-well plates in growth medium and after an overnight attachment period were exposed to selected inhibitor concentrations or vehicle for 24 h. The medium was aspirated and cells were washed with inhibitor-free medium. Cells were allowed to grow for an additional 10 days. All studies were performed in triplicate.

2.5. Western blotting analysis

Total cell lysates were prepared and analysed by Western Blot analysis as described previously.[4] Equal amounts of protein

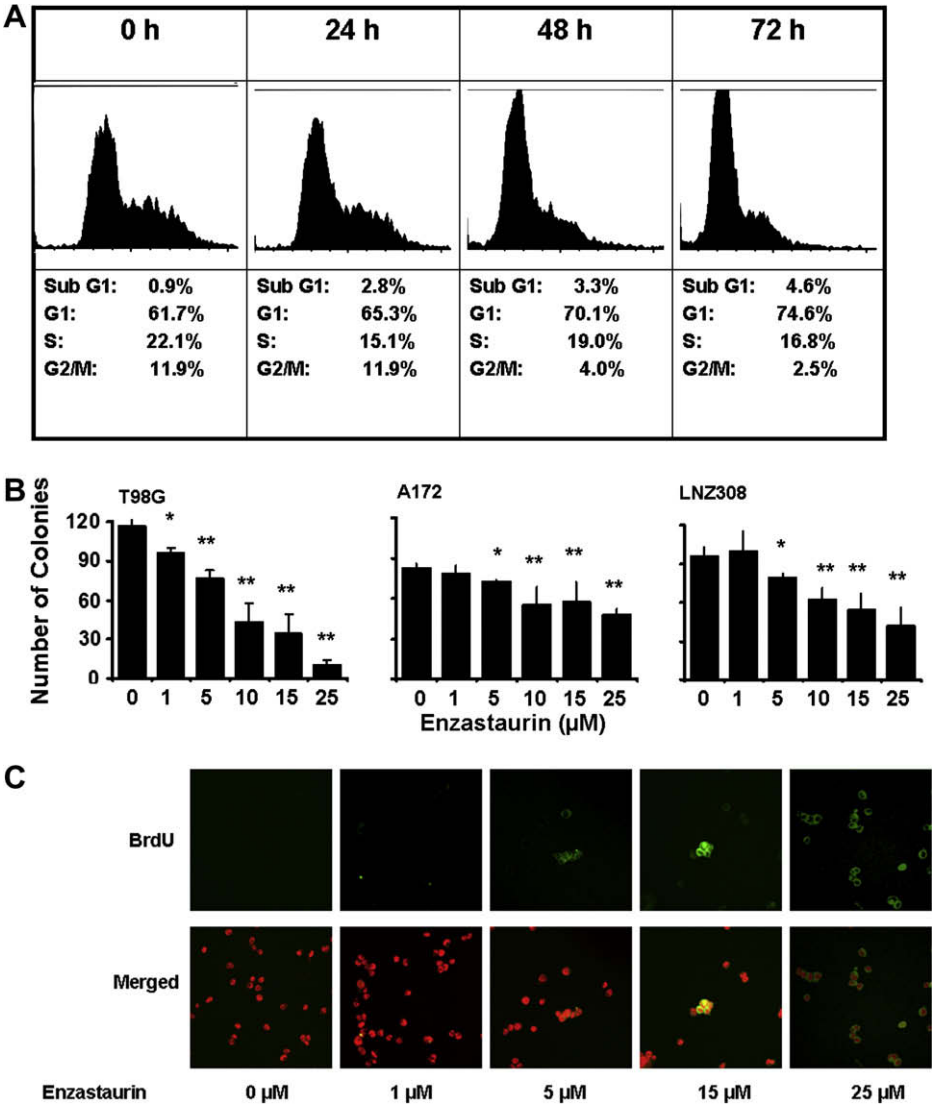

Fig. 1 – The effect of enzastaurin on cell cycle distribution and clonogenicity. (A) T98G cells were treated with 5 μM of enzastaurin and fixed at different time points. Cell cycle progression was evaluated via flow cytometry. The effect of treatment on cell cycle phase distribution in T98G cells is summarised below in each histogram. Following exposure to enzastaurin, a distinct G_1 cell cycle block with a concomitant decrease of those cells in S and G_2/M phase was demonstrated. A fraction of sub-G_1 (apoptotic) cells was also observed. Ten thousand cells were counted per experimental condition. The experiments were repeated. A representative flow cytometry histogram is shown. (B) Human glioma cell lines T98G, A172 and LNZ308 were exposed to varying concentrations of enzastaurin for 24 h. The following day, the media were changed, and complete media were added. Cells were grown for an additional 10 days in the absence of inhibitor, and colonies were counted. Points represent the mean of two experiments ± standard deviation. $^{*}P < 0.01$ versus control. $^{**}P < 0.001$ versus control. (C) T98G cells were treated with different concentrations of enzastaurin for 24 h and then were fixed and analysed with APO-BrdU TUNEL assay kit. As shown in the figures, Br-dUTP was more strongly incorporated into cells treated with higher concentrations of enzastaurin.

were separated by SDS–polyacrylamide gel electrophoresis and electro transferred onto a nylon membrane (Invitrogen, Carlsbad, CA). Primary antibodies were purchased from Cell Signalling Technology and used according to the manufacturer's recommendations. Secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The proteins were visualised by enhanced chemiluminescence (Cell Signaling Technology). Where indicated, the blots were reprobed with antibodies against β-actin to ensure equal loading and transfer of proteins. Relative reactivities of proteins on immunoblots were quantified in digitised bands of chemiluminescence with correction for background.

2.6. *Immunocytochemistry and fluorescence microscopy*

Cells were grown on chamber slides (Nalge Nunc, Naperville, IL) in growth medium, and after an overnight attachment period were exposed to selected concentrations of inhibitor or vehicle (DMSO) for various durations. Cells were fixed with 3.7% formaldehyde for 15 min, washed in PBS and

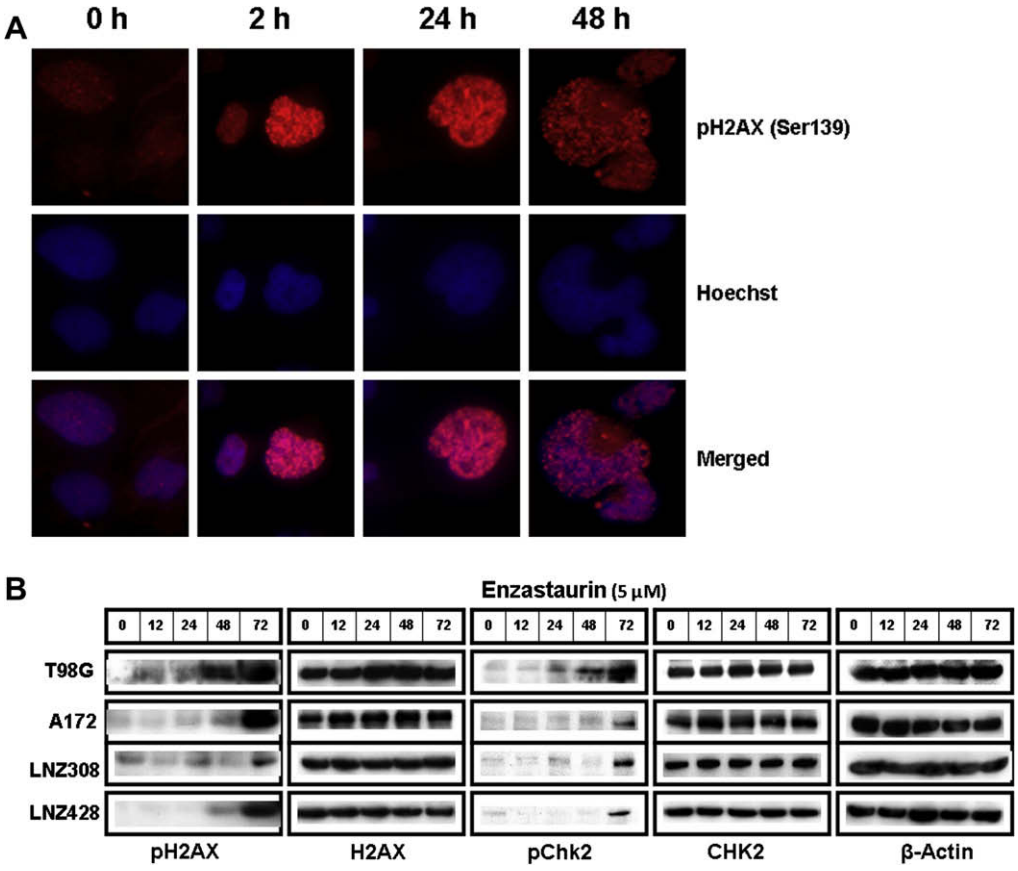

Fig. 2 – Enzastaurin induces H2AX and Chk2 phosphorylation. (A) Logarithmically growing T98G cells were incubated with 5 μM of enzastaurin and cells were fixed, permeabilised and stained with an antibody to p-H2AX (red) to visualise focus formation. Distinct phosphorylated H2AX foci were evident in the nuclei of T98G cells within 2 h of exposure to 5 μM enzastaurin. (B) Whole cell extracts were prepared from T98G, A172, LNZ428 and LNZ308 cells after exposure to 5 μM enzastaurin for varying durations and analysed by immunoblotting using phospho-H2AX (Ser139) and phospho-Chk2 (Thr68) antibodies. Blots were stripped and reprobed with an antibody against total H2AX, Chk2 and β-actin. Enzastaurin-induced Chk2 activation and H2AX phosphorylation were seen in a time-dependent fashion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

permeabilised with 0.1% Triton X-100 in PBS for 15 min. After blocking with 0.3% bovine serum albumin and 1% goat serum for 1 h, cells were incubated with an antibody against phospho-Histone H2AX (rabbit) (1:100, incubated overnight at 4 °C). After PBS wash, the slides were incubated with the secondary antibody (TRITC-goat anti-rabbit; Invitrogen) for 1 h at room temperature. To visualise apoptosis-induced DNA fragmentation, enzastaurin-treated cells were stained using the APO-BrdU TUNEL Assay Kit protocol (Invitrogen), which detects incorporation of BrdU into the DNA of genomic DNA disrupted by cellular nucleases. Cells were then washed, mounted and examined under an Olympus IX81 confocal microscope and imaged using the Olympus Fluoview software (Version 1.5).

3. Results

3.1. *Enzastaurin induces both cell cycle arrest and apoptosis*

We[3] and others[21,22] have shown that enzastaurin induces apoptosis in malignant human glioma cell lines in a dose- and time-dependent manner. To further study the effects on cell cycle progression and apoptosis, T98G cells were exposed to 5 μM enzastaurin for various intervals and examined by flow cytometry. Treatment with enzastaurin did not markedly affect the cell cycle distribution during short periods of time (6 and 12 h, data not shown), although with longer exposures enzastaurin induced accumulation of cells in G1 phase in a time-dependent (Fig. 1A) manner, with a concomitant decline in the percentage of cells in S and G2/M phase relative to controls. Increase in the sub-G0 fraction was also observed, consistent with induction of apoptosis. The cytotoxic effect of enzastaurin was further confirmed using a clonogenic assay. T98G, A172 and LNZ308 cells were treated with varying concentrations of enzastaurin for 1 day, medium was aspirated and the cells were washed with inhibitor-free medium. Cells were allowed to grow for an additional 10 days. There was a dose-dependent decrease in colony forming ability due to enzastaurin (Fig. 1B). These results suggest that the decreased number of colonies observed after treating with enzastaurin is at least partly due to cell cycle arrest and/or induction of apoptosis. To assess apoptosis induced by enzastaurin (Fig. 1C), T98G cells were treated with a range of concentra-

tions of enzastaurin for 24 h. This analysis demonstrated a concentration-dependent increase in apoptotic labelling, most apparent with concentrations >5 μM.

3.2. *Enzastaurin induces H2AX and Chk2 phosphorylation*

To examine the mechanisms involved in propagating and executing apoptosis in response to enzastaurin in glioma cells, T98G cells were exposed to enzastaurin followed by assessment of H2AX focus formation, a sensitive and selective signal for the existence of DSB,[23,24] by immunofluorescent detection of Ser^{139} H2AX phosphorylation. As shown in Fig. 2A, distinct phosphorylated H2AX foci were evident in the nucleus of T98G cells within 2 h of exposure to 5 μM enzastaurin. Because Chk2 is important for the transduction of DNA damage signals, we investigated if Chk2 phosphorylation was also associated with enzastaurin-induced apoptosis. Whole cell extracts were prepared from T98G, A172, LNZ308 and LNZ428 cells after exposure to 5 μM enzastaurin for varying durations for Western blot analysis. These studies demonstrated enzastaurin-induced Chk2 activation and H2AX phosphorylation, suggesting that the mechanism of enzastaurin-induced apoptosis involves induction of DNA damage pathways (Fig. 2B).

3.3. *MAPK family inhibition counteracts enzastaurin-induced Chk2 and H2AX phosphorylation and PARP cleavage*

To elucidate signal transduction pathways responsible for the regulation of H2AX and Chk2 phosphorylation induced by enzastaurin, we examined the potential involvement of MAPK family members by Western blot analysis. The data indicated that enzastaurin induced the phosphorylation of ERK (Thr202/Tyr204), p38 (Thr180/Tyr182) and JNK (Thr183/Tyr185) in a time-dependent manner (Fig. 3A–C). Phosphorylation of ERK was induced in response to enzastaurin with exposure intervals between 3 and 24 h (Fig. 3A), with an even more striking induction of p38 activation (Fig. 3B) and a smaller increase in JNK phosphorylation (Fig. 3C).

To determine whether MAPK family members were involved in the regulation of H2AX and Chk2 phosphorylation or, conversely, represented a compensatory mechanism that allowed cells to resist apoptosis induction, we examined the effect of blocking these pathways using the chemical inhibitors U0126 (MEK inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor). Cells were pretreated with U0126 (Fig. 4A), SB203580 (Fig. 4B) or SP600125 (Fig. 4C) in the culture media 60 min prior to treatment with 5 μM enzastaurin for 24 h. Total proteins were then extracted for Western blot analysis. As expected, enzastaurin induced a strong activation of H2AX and Chk2. However, all three MAPK inhibitors completely abolished enzastaurin-induced H2AX and Chk2 phosphorylation, suggesting that enzastaurin-induced H2AX and Chk2 phosphorylation can be regulated by ERK, p38 or JNK pathways (Fig. 4A–C).

To determine whether MAPK family blockade also attenuated apoptosis induction in response to enzastaurin, T98G cells were exposed to enzastaurin with or without MAPK family inhibitors as noted above, and Western immunoblot analysis was performed using an antibody against cleaved PARP. These studies demonstrated that enzastaurin-induced PARP cleavage was significantly reduced when the cells were pretreated with U0126, or SB203580, or SP600125 (Fig. 4D). Our observations clearly suggest that enzastaurin-induced apoptosis was regulated by MAPK pathways and inhibitors of MAP kinases protect malignant human glioma cells from apoptosis, rather than potentiating the efficacy of enzastaurin. This was confirmed by cell proliferation assessed by MTS assay after 72 h (Fig. 4E). T98G and U87 cells showed significantly reduced cell death when pretreated with MAPK family inhibitors than cells treated with enzastaurin alone.

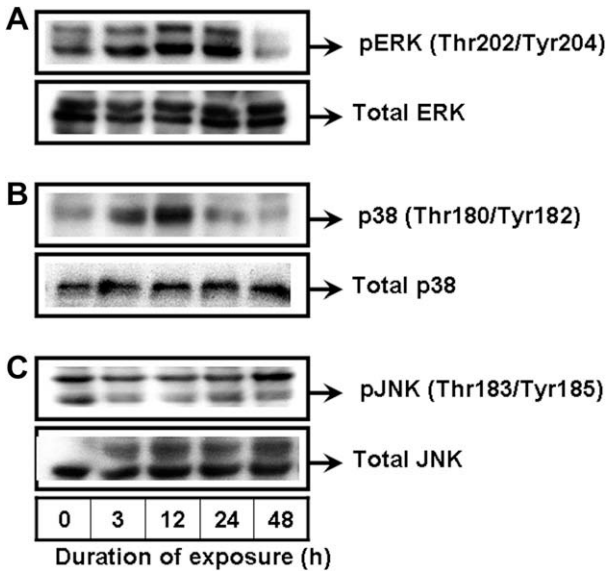

Fig. 3 – Enzastaurin induces MAPK family phosphorylation. T98G cells were treated with 5 μM of enzastaurin and total proteins were extracted at the indicated time points for Western blot analysis. The data indicated that enzastaurin induced the phosphorylation of ERK (A), p38 (B) and JNK (C) in a time-dependent manner.

4. Discussion

Enzastaurin is a novel PKC-β inhibitor with anti-tumour activity that is currently under clinical investigation in several tumour entities, including glioblastoma.[25] Several mechanisms by which enzastaurin exerts its anti-tumour effects have been described. First, enzastaurin has been shown to have anti-angiogenic properties associated with decreased VEGF levels.[26] Supporting these observations, anti-glioma activity of enzastaurin has been shown in a mouse xenograft model.[27] It was also observed that treatment with enzastaurin resulted in synergistic activity with irradiation and reduced expression of VEGF in vitro and in vivo leading to reduced vessel density in glioma xenografts. Second, enzastaurin has direct cell death-inducing properties in tumour cells *in vitro*.[28] Recently, we and others have shown that treatment with enzastaurin was associated with reduced phosphorylation of Akt in several tumour cell lines,[3,28–31] although the exact mechanism(s) underlying enzastaurin-dependent cell death induction have remained unclear.

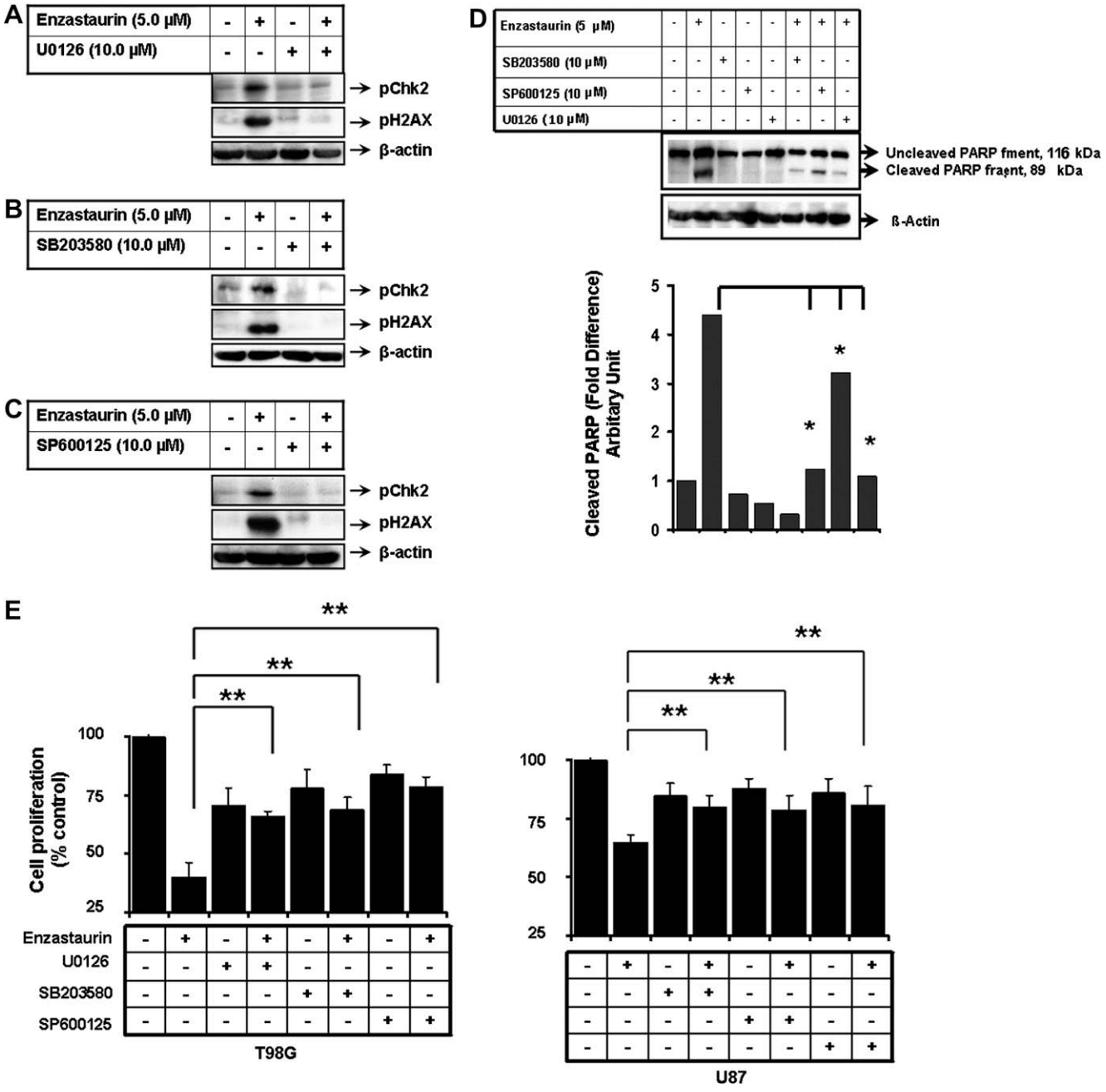

Fig. 4 – MAPK family inhibition counteracts enzastaurin-induced Chk2 and H2AX phosphorylation and PARP cleavage. T98G cells were pretreated with U0126 (A), SB203580 (B) or SP600125 (C) in the culture media 60 min prior to treatment with 5 μM enzastaurin for 24 h. Total proteins were extracted for Western blot analysis. Enzastaurin induced a strong activation of H2AX and Chk2. All three MAPK inhibitors completely abolished enzastaurin-induced H2AX and Chk2 phosphorylation. (D) T98G cells were pretreated with SB203580 or U0126 or SP600125 in the culture media 60 min prior to treatment with 5 μM enzastaurin for 24 h. Western immunoblot analysis was performed using an antibody against cleaved PARP. Ratios of cleaved PARP in the lysates of cells treated with inhibitors, with and without enzastaurin are shown on the bottom panel. (E) T98G and U87 cells were pretreated with SB203580 or U0126 or SP600125 in the culture media 60 min prior to treatment with 5 μM enzastaurin for 72 h. Cell numbers were assessed semi-quantitatively by spectrophotometric measurement of MTS bioreduction. Points represent the mean of three measurements ± standard deviation ($P < 0.001$ versus enzastaurin alone). This clearly demonstrates that the inhibition of MAPK family members diminishes cell death induced by enzastaurin alone.**

There is compelling evidence in the literature that H2AX phosphorylation is a highly specific marker of the presence of DSBs in nuclear chromatin.[32] Induction of DSBs, whether generated by ionising radiation or by DNA-cleaving drugs such as topo1 and topo2 inhibitors, activates ATM, which in turn phosphorylates H2AX and Chk2.[33,34] Our results suggest that H2AX and Chk2 phosphorylation is also required for enzastaurin-induced apoptosis.

In addition, we found that activation of MAPK pathways played an important role in enzastaurin-induced apoptosis in human malignant glioma cell lines. The MAPK signalling pathways have multiple roles in natural processes such as cell growth, differentiation, cytoskeleton dynamics and apoptosis.[35] In a previous report, we[3] showed that exposure of malignant human glioma cells to enzastaurin induces caspase-3 activation, Bax cleavage and apoptosis. In agreement with recent reports,[22] the current study demonstrated that exposure of T98G to this agent led to a significant level of MAPK activation in a dose- and time-dependent manner, strongly supporting the involvement of MAPK family members in the transmission of enzastaurin-induced stress responses in glioma cells.

A number of studies have reported on the role of MAPK family members in coupling cellular stress signals leading to apoptotic cell death. The functional role of MAPK in cellular stress response is, however, less well understood and likely varies significantly in normal and neoplastic cell types.[36,37] Our observation that H2AX phosphorylation and apoptosis induction was suppressed by pretreatment of cells with chemical inhibitors of MAPK family kinases suggests that MAPK activation represents a critical step in mediating apoptotic signalling in glioma cells in response to enzastaurin, rather than a compensatory adaptation by the cells to achieve resistance to this agent.

This observation is consistent with recent findings that suggest the involvement of MAPK family/H2AX interactions in mediating apoptosis induced by a variety of stimuli. Specifically, it was shown that ultraviolet A-irradiation activates JNK, which phosphorylates H2AX to regulate apoptosis, whereas serum starvation has been noted to induce p38 phosphorylation as a precursor to H2AX activation, without affecting the phosphorylation of ERK or JNK.[38,39] Thus, different stress stimuli may regulate cellular apoptosis by different pathways which require H2AX phosphorylation as a common mediator.

Taken together, the current studies provide strong evidence that apoptosis induced by enzastaurin is MAPK-dependent, and that H2AX and Chk2 phosphorylation is implicated in this process, mediated by MAPK family kinases. The balance between cell survival and cell death is a complex issue, and there is considerable effort to understand how tumour cells regulate the decision points between these critical pathways and to tip the balance in favour of apoptosis. Given the relatively modest clinical efficacy that has been observed with single agent molecularly targeted approaches, including enzastaurin, in gliomas, there is a strong rationale for efforts directed at identifying logical therapeutic combinations. In this regard, our previous studies demonstrated synergistic activity between enzastaurin and the heat shock protein inhibitor, 17-AAG. Although one might predict that combining a PKC inhibitor, such as enzastaurin, with MAPK inhibition might lead to potentiation of apoptosis induction, the results of the current study suggest that, to the contrary, such combinations may actually lead to antagonistic effects. These observations provide new insights into the mechanisms involved in the induction of apoptosis by enzastaurin in malignant human glioma cell lines and identify a novel pathway mediated by the activation and phosphorylation of H2AX and Chk2, which significantly contributes to the induction of the apoptotic process.

Conflict of interest statement

None declared.

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