Chemosensitization of glioblastoma cells by the histone deacetylase inhibitor MS275

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Glioblastoma is the most common primary brain tumor with a dismal prognosis, highlighting the need for novel treatment strategies. Here, we provide the first evidence that the histone deacetylase inhibitor, MS275, sensitizes glioblastoma cells for chemotherapy-induced apoptosis. Pretreatment of glioblastoma cells with MS275 causes acetylation of histone H3 protein and significantly enhances doxorubicin-induced apoptosis. Calculation of combination index showed that MS275 and doxorubicin acted in a synergistic manner to trigger apoptosis. Furthermore, pre-exposure to MS275 significantly increases apoptosis in response to temozolomide, etoposide, and cisplatin. In contrast, treatment with MS275 before the addition of vincristine and taxol significantly reduces the induction of apoptosis. Analysis of cell cycle alterations showed that treatment with MS275 triggers G1 cell cycle arrest, which in turn renders cells less sensitive to the cytotoxic effects of mitotic inhibitors, such as vincristine and taxol. Thus, these findings show for the first time that the histone deacetylase inhibitor, MS275, represents a promising strategy to prime glioblastoma cells for chemotherapy-induced apoptosis in a drug-specific manner. *Anti-Cancer Drugs* 22:494–499 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Glioblastoma is the most frequent malignant primary brain tumor that bears a very poor prognosis [1]. The median survival of patients is less than 15 months after primary diagnosis, despite multimodal treatment regimens [2]. This highlights the need to develop novel and more effective treatment strategies.

Most current cytotoxic cancer therapies primarily act by inducing apoptosis (programmed cell death) in cancer cells [3]. For example, chemotherapeutic drugs have been shown to initiate the intrinsic (mitochondrial) pathway of apoptosis. This leads to the release of apoptogenic factors such as cytochrome c from the mitochondrial intermembrane space into the cytosol where caspase-9 and caspase-3 become activated [4]. Apoptosis is tightly controlled by various proapoptotic and antiapoptotic proteins [5]. Evasion of apoptosis is a hallmark of human cancers [6], and can also confer resistance to anticancer therapy [5]. Thus, lowering the threshold for apoptosis induction presents an attractive approach to overcome chemoresistance.

Sensitivity to apoptosis can be restored in combination therapies, for example, together with histone deacetylase inhibitors (HDACI) [7]. HDACI can revert aberrant epigenetic states in cancer cells, which result from deregulated expression or activity of histone deacetylases (HDACs) and histone acetyl transferases [8]. These enzymes determine the acetylation status of histones, thereby affecting chromatin topology and gene expression [9]. In addition to histones, HDACs can target many nonhistone proteins [10]. HDACIs have been described to induce differentiation, cell cycle arrest, and apoptosis in various types of cancer [11]. MS275 (entinostat) is a benzamide derivative that is currently under evaluation in phase I and II clinical trials for the treatment of several solid and hematological malignancies [8,12].

Searching for strategies to enhance chemosensitivity of glioblastoma cells, we investigated in this study whether the HDACI MS275 is capable of increasing chemotherapy-induced apoptosis in glioblastoma.

Materials and methods

Cell culture and reagents

Glioblastoma cell lines were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and maintained in Dulbecco's modified Eagle's medium medium (Life Technologies, Eggenstein, Germany), supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 1 mmol/l L-glutamine (Invitrogen, Karlsruhe, Germany), 1% penicillin/streptomycin (Invitrogen), and 25 mmol/l HEPES (Biochrom) as described previously [13]. MS275 was kindly provided by Bayer Schering (Berlin, Germany), and all other chemicals were

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purchased from Sigma (Deisenhofen, Germany), unless otherwise stated.

For experiments, cells were plated at 0.4×10^5 cells/cm^2 in 24-well plates (determination of apoptosis, cell cycle analysis) or in 10 cm plates (western blotting). Cells were treated with MS275 for 24 h, followed by the addition of chemotherapeutic agents at indicated time points in the presence of MS275.

Determination of apoptosis and cell cycle analysis

For the determination of apoptosis, nonadherent and adherent cells were collected. Apoptosis was determined by fluorescence-activated cell sorting analysis (FACScan, BD Bioscience, Heidelberg, Germany) of DNA fragmentation of propidium iodide-stained nuclei as described previously [14]. Specific apoptosis (percentage) was calculated as follows: 100 × (percentage of apoptotic cells in a sample – mean percentage of spontaneous apoptosis in controls)/(100 – mean percentage of spontaneous apoptosis in controls). For cell cycle analysis, adherent cells were collected. Cell cycle analysis was carried out in permeabilized cells stained with propidium iodide using ModFitLT V3.2.1 program (BD Biosciences).

Western blot analysis

Western blot analysis was carried out using 50 μg of protein per lane as described previously [14]. The following antibodies were used: acetylated histone H3 (Upstate Biotechnology, Lake Placid, New York, USA) and α-tubulin (Calbiochem, San Diego, California, USA). Goat antimouse immunoglobulin G and goat antirabbit immunoglobulin G conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, California, USA) were used as secondary antibodies. Enhanced chemiluminescence was used for detection of antibody reaction (Amersham Bioscience, Freiburg, Germany).

Statistical analysis

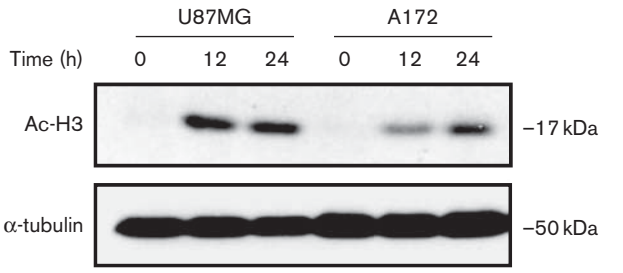
Statistical significance was assessed by Student's *t*-test (two-tailed distribution, two-sample, unequal variance). Drug interaction was analyzed by the combination index (CI) method using CalcuSyn software (Biosoft, Cambridge, UK). CI less than 0.9 indicates synergism, 0.9–1.1 indicates additivity, and greater than 1.1 indicates antagonism.

Results and discussion

MS275 causes acetylation of histone H3 in glioblastoma cells

Initially, we performed control experiments to monitor histone acetylation upon treatment with the HDACI, MS275. Treatment of U87MG and A172 glioblastoma cells with MS275 caused acetylation of histone H3 (Fig. 1).

Fig. 1



MS275 causes histone acetylation in glioblastoma cells. U87MG and A172 glioblastoma cells were treated for the indicated times with 8 μmol/l MS275 (U87MG) or 5 μmol/l MS275 (A172). Protein expression of acetylated histone H3 and α-tubulin was assessed by western blotting.

MS275 sensitizes glioblastoma cells for doxorubicin-induced apoptosis in a synergistic manner

To investigate whether HDAC inhibition is a suitable strategy to enhance chemosensitivity of glioblastoma cells, we assessed the effect of MS275 on doxorubicin-induced apoptosis. Importantly, pretreatment for 24 h with a subtoxic dose of MS275 (that triggered less than 20% apoptosis) significantly increased doxorubicin-induced apoptosis in U87MG and A172 glioblastoma cells when compared with treatment with doxorubicin alone (Fig. 2). This MS275-mediated sensitization toward doxorubicin occurred in a dose-dependent and time-dependent manner (Fig. 2). To determine whether the interaction of MS275 and doxorubicin is additive or synergistic, we calculated the CI. Of note, MS275 synergistically increased doxorubicin-triggered apoptosis (Fig. 3, Table 1).

MS275 enhances temozolomide-induced apoptosis in glioblastoma cells

Next, we tested the effect of MS275 on apoptosis sensitivity of glioblastoma cells toward temozolomide, the most frequently used anticancer drug in clinical protocols for glioblastoma. As temozolomide-induced apoptosis has been described to occur in a delayed manner [15], we used longer incubation times of 96 and 120 h with temozolomide and accordingly lower concentrations of MS275 compared with the previous experiments with doxorubicin. Importantly, pre-exposure of glioblastoma cells to MS275 significantly increased temozolomide-induced apoptosis in a dose-dependent manner compared with single-agent treatment with temozolomide (Fig. 4).

MS275 sensitizes glioblastoma cells for etoposide-induced and cisplatin-induced apoptosis, whereas it reduces microtubule inhibitor-mediated apoptosis

To further explore whether MS275 globally alters apoptosis sensitivity of glioblastoma cells, we extended our study to additional anticancer drugs. Pretreatment with MS275 significantly increased apoptosis, after the

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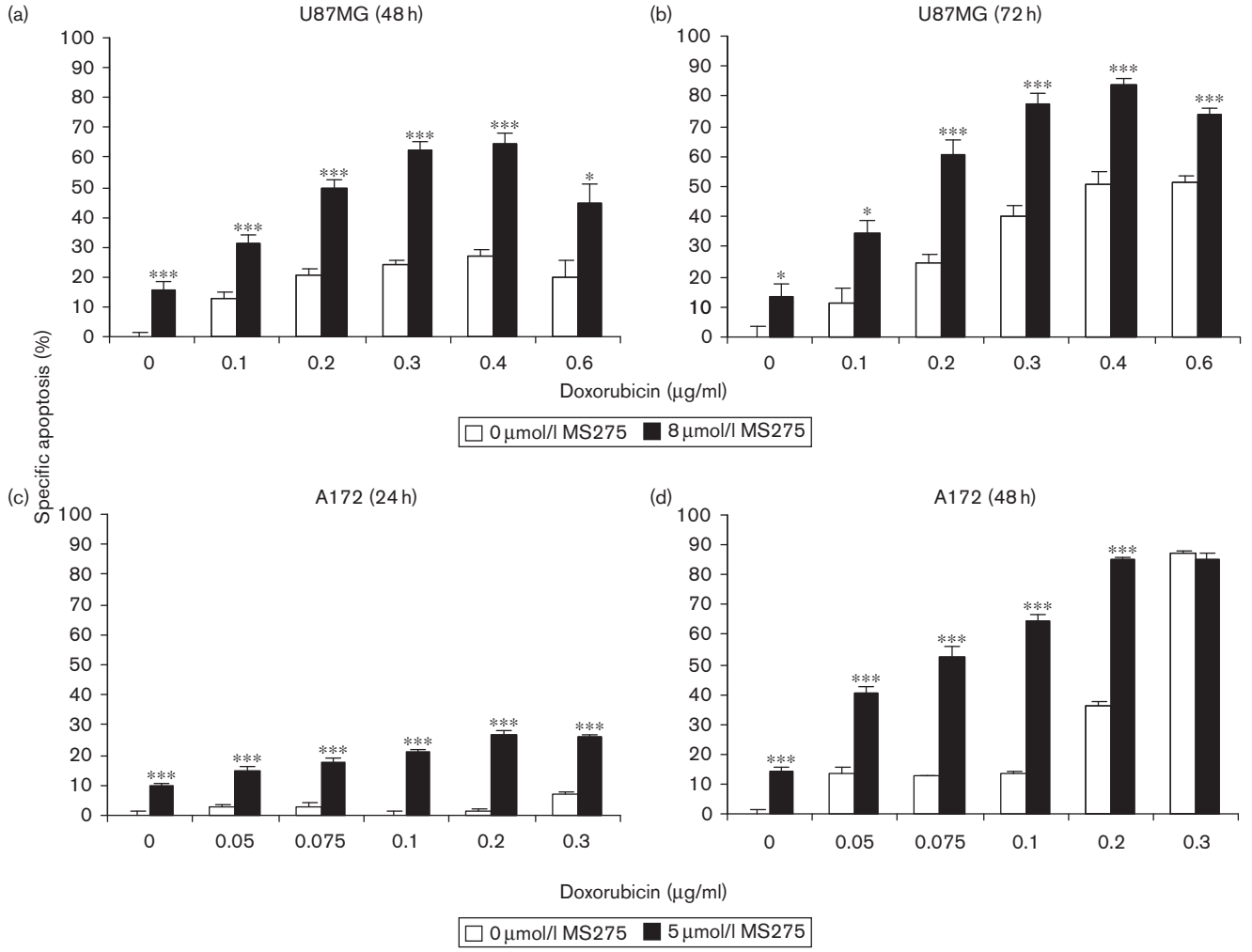
Fig. 2



MS275 sensitizes glioblastoma cells for doxorubicin-induced apoptosis. U87MG (a, b) and A172 (c, d) glioblastoma cells were treated for 24 h with (black bars) or without (white bars) MS275 (U87MG: 8 μmol/l, A172: 5 μmol/l), followed by the addition of indicated concentrations of doxorubicin for indicated times. Apoptosis was determined by fluorescence-activated cell sorting analysis of DNA fragmentation of propidium iodide-stained nuclei. Mean + standard error of the mean of at least three independent experiments in duplicate are shown; $*P < 0.05$, $***P < 0.001$ comparing samples in the presence and absence of MS275.

addition of etoposide or cisplatin (Fig. 5a and b). In contrast, the induction of apoptosis by vincristine or taxol, two microtubule inhibitors, was significantly reduced in the presence of MS275 (Fig. 5c and d). These findings indicate that MS275 sensitizes glioblastoma cells for chemotherapeutic agents in a stimulus-specific manner.

To investigate the possible underlying mechanisms for this observed antagonistic interaction of MS275 with microtubule disrupting agents, we carried out cell cycle analysis. Interestingly, treatment with MS275 caused a significant increase of cells in G1 phase accompanied by a significant decrease in S and G2 phases of the cell cycle (Fig. 5e). This suggests that MS275-mediated cell cycle arrest at G1 may render cells less sensitive to mitotic inhibitors.

In this study, we show for the first time that the HDAC inhibitor, MS275, primes glioblastoma cells for chemotherapy-induced apoptosis. Of note, this MS275-mediated sensitization for chemotherapy occurs in a drug-specific manner. Accordingly, pretreatment with MS275 markedly increases apoptosis induced by temozolomide, doxorubicin, etoposide, and cisplatin. In contrast, apoptosis in response to vincristine and taxol is significantly decreased in the presence of MS275. Vincristine and taxol are both microtubule-disrupting agents that either inhibit microtubule polymerization (vinca alkaloids such as vincristine) or stabilize microtubule formation (taxanes such as taxol) [16]. As vincristine and taxol exert their anticancer activity by inhibiting mitosis, cycling cells are more vulnerable to mitotic inhibitors. As we observed that pretreatment with

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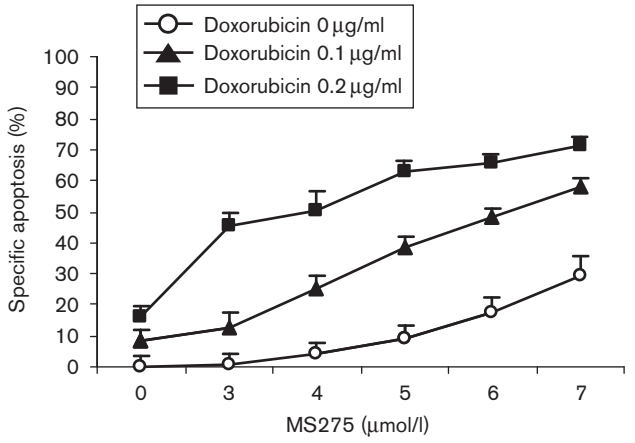
MS275 causes cell cycle arrest in the G1 phase with a concomitant decrease of cells in S and G2 phases of the cell cycle, the observed antagonistic interaction of MS275 and vincristine or taxol may be due to MS275-mediated G1 arrest, a phase of the cell cycle in which cells are less sensitive to mitotic inhibition.

Although our study is the first to show that MS275 chemosensitizes glioblastoma cells, vorinostat, another HDACI, has previously been described to enhance the cytotoxic effects of the topoisomerase I inhibitor, SN38, in glioblastoma cells [17]. Furthermore, the HDACIs trichostatin A and phenylbutyrate have been reported to sensitize glioblastoma cells to ionizing radiation [18,19]. Together, these data underscore the potential of HDACI to increase chemosensitivity or radiosensitivity in glioblastoma.

Our findings have important clinical implications for the development of novel treatment approaches of glioblastoma, the most aggressive primary brain tumor that is still incurable. Our data propose MS275 as a potentially

Fig. 3



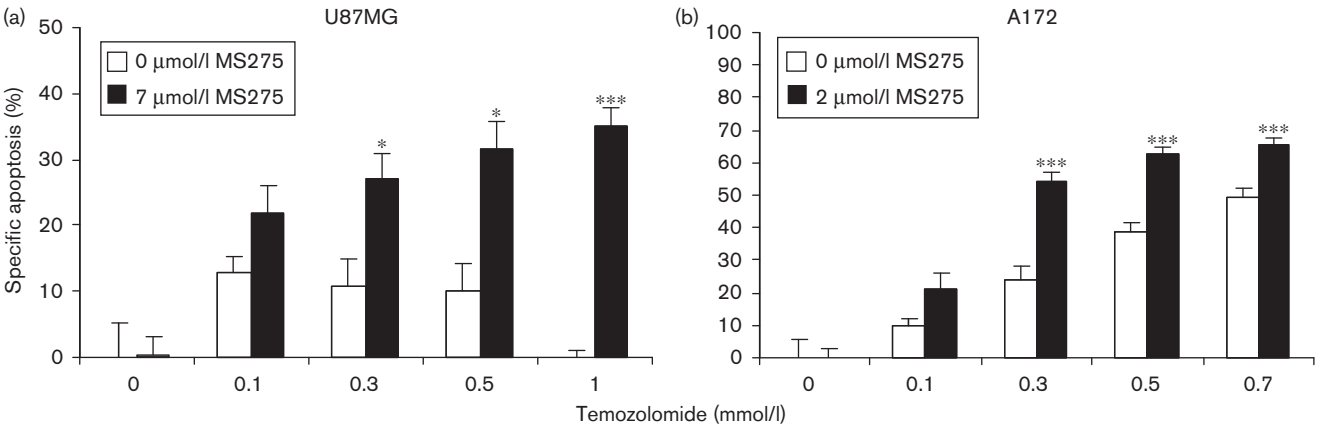
Synergistic induction of apoptosis by MS275 and doxorubicin. A172 glioblastoma cells were treated for 24 h with or without indicated concentrations of MS275, followed by the addition of indicated concentrations of doxorubicin for 48 h. Apoptosis was determined by fluorescence-activated cell sorting analysis of DNA fragmentation of propidium iodide-stained nuclei. Mean of at least three independent experiments in duplicate are shown.

Table 1 Synergistic induction of apoptosis by MS275 and doxorubicin

Doxorubicin (µg/ml)	MS275 (µmol/l)	CI value
A		
0.1	3	1.219
0.1	4	0.891
0.1	5	0.820
0.1	7	0.841
0.2	3	0.612
0.2	4	0.674
0.2	5	0.641
0.2	7	0.756
B		
0.1	4	0.740
0.1	5	0.478
0.1	6	0.350
0.1	8	0.238
0.2	4	0.831
0.2	5	0.580
0.2	6	0.415
0.2	8	0.238

Combination index (CI) was calculated as described in Materials and methods section for apoptosis induced by combined treatment of A172 (A) or U87MG (B) cells treated for 24 h with or without indicated concentrations of MS275, followed by the addition of indicated concentrations of doxorubicin for 48 h. CI values of <0.9 indicates synergism, $>0.9-<1.1$ additivity, >1.1 antagonism.

Fig. 4



MS275 sensitizes glioblastoma cells for temozolomide-induced apoptosis. U87MG (a) and A172 (b) glioblastoma cells were treated for 24 h with (black bars) or without (white bars) MS275 (U87MG: 7 µmol/l; A172: 2 µmol/l), followed by the addition of indicated concentrations of temozolomide for 120 h (a) or 96 h (b). Apoptosis was determined by fluorescence-activated cell sorting analysis of DNA fragmentation of propidium iodide-stained nuclei. Mean + standard error of the mean of at least three independent experiments in duplicate are shown; $*P<0.05$, $***P<0.001$ comparing samples in the presence and absence of MS275.

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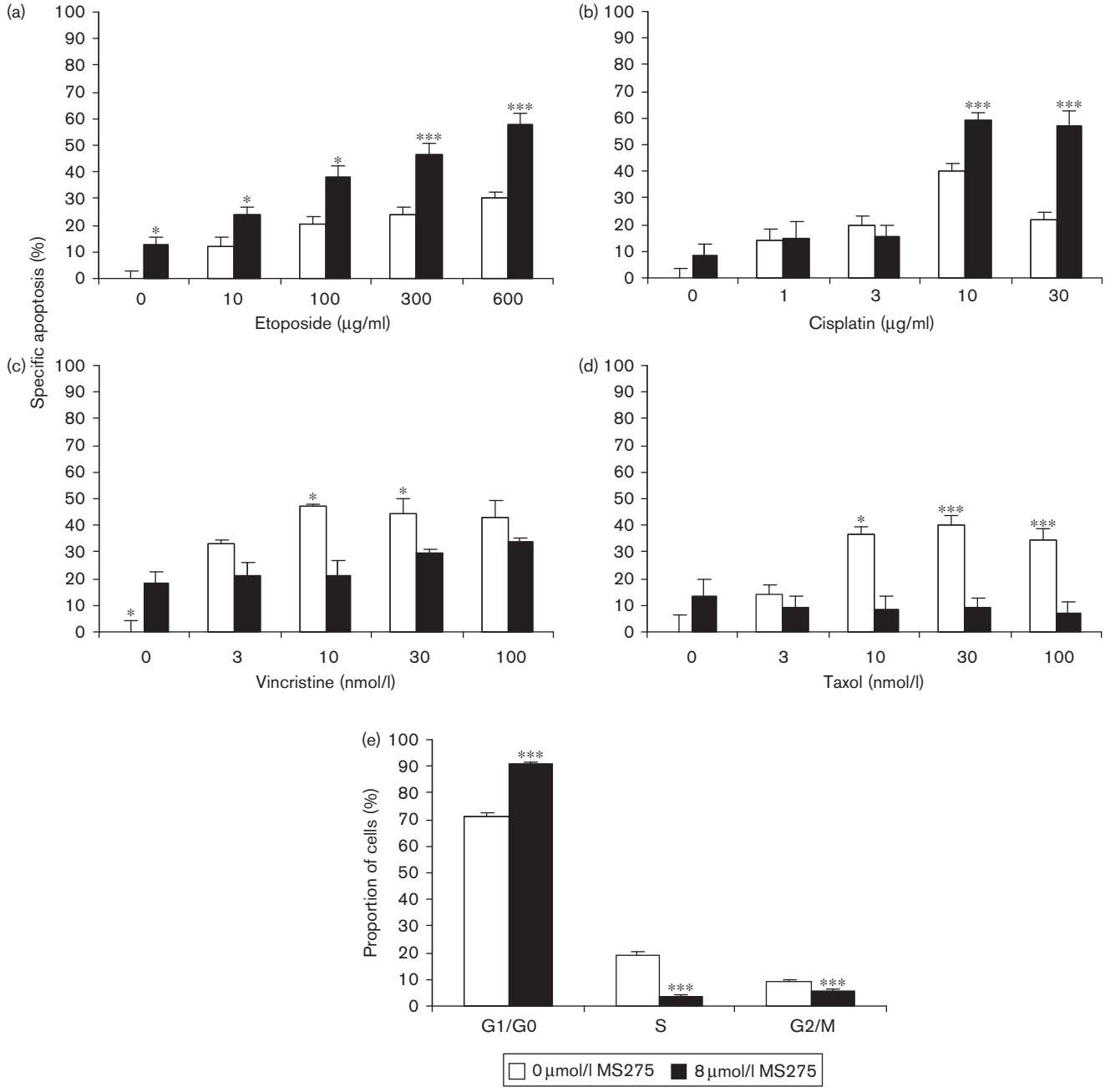
Fig. 5



MS275 enhances etoposide-triggered or cisplatin-triggered apoptosis, but reduces vincristine-induced or taxol-induced apoptosis. U87MG cells were treated for 24 h with (black bars) or without (white bars) 8 μmol/l MS275, followed by the addition of indicated concentrations of etoposide for 48 h (a), cisplatin for 72 h (b), vincristine for 72 h (c), or taxol for 72 h (d). Apoptosis was determined by fluorescence-activated cell sorting analysis of DNA fragmentation of propidium iodide-stained nuclei. In (e), U87MG cells were treated for 30 h with (black bars) or without (white bars) 8 μmol/l MS275, and cell cycle analysis was carried out as described in Materials and methods section. Mean + standard error of the mean of at least three independent experiments in duplicate (a–d) or triplicate (e) are shown; $*P < 0.05$, $***P < 0.001$ comparing samples in the presence and absence of MS275.

suitable combination partner for certain anticancer drugs, such as temozolomide, doxorubicin, etoposide, and cisplatin, to trigger apoptosis. The use of MS275 in glioblastoma therapy is encouraged by studies showing the ability of MS275 to cross the blood–brain barrier [20,21]. MS275 is already in phase I and II clinical trials for the treatment of several solid and hematological malignancies [8], underscoring the feasibility to translate this combination strategy of MS275 and chemotherapy into a clinical application in glioblastoma. Thus, our

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findings have important implications for the development of novel treatment strategies in glioblastoma.

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