Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2008 | 107 | 616–627 doi: 10.1111/j.1471-4159.2008.05625.x

Involvement of miltefosine-mediated ERK activation in glioma cell apoptosis through Fas regulation

Richa Tewari, Vivek Sharma, Nitin Koul and Ellora Sen

National Brain Research Centre, Manesar, Haryana, India

Abstract

The anti-neoplastic property of alkyl phospholipids has been tested for the treatment of several malignancies. In this study, we evaluated the efficacy of miltefosine (Hexadecylphosphocholine – an alkyl phospholipids analogue) on glioblastoma multiforme. In this study, we demonstrate that miltefosine-induced apoptosis is accompanied by elevated Fas, Fas-associated death domain (FADD) expression, caspase-8 activity and the increased distribution of Fas and FADD towards lipid raft microdomain to form death inducing signaling complex. Treatment with miltefosine resulted in increase in Ras, extracellular signal-regulated kinase (ERK) and p38MAPK activity. Expression of dominant-negative Ras (Ras N17) attenuated miltefosine-mediated apoptosis. Although inhibition of both ERK and p38MAPK decreased the pro-apoptotic effects of miltefosine, it was the inhibition of ERK and not p38MAPK activation that decreased Fas and FADD expression. An ERK-dependent increase in the expression of γH2AX-involved in response to DNA double-stranded breaks was also observed. Taken together, our findings suggest the involvement of ERK activation in miltefosine-induced glioma cell apoptosis.

Keywords: apoptosis, Fas, glioblastoma, lipid rafts, miltefosine, Ras.

J. Neurochem. (2008) **107**, 616–627.

Glioblastoma multiforme (GBM) remains one of the most challenging solid cancers to treat because of its highly proliferative, angiogenic and invasive nature (DeAngelis 2001). Although Fas, a member of the tumor necrosis factor family involved in signaling apoptotic cell death in susceptible target cells (Wallach *et al.* 1999) is expressed in majority of glioblastoma (Tachibana *et al.* 1995); GBMs are resistant to death from Fas pathway activation (Xia *et al.* 2005). Therefore, inducing GBM cell death by targeting death receptor pathways is considered as an attractive therapy (Xia *et al.* 2005). Fas has the ability to interact with Fas-associated death domain (FADD) directly which recruits procaspase-8 to form death-inducing signaling complex (DISC) (Kischkel *et al.* 1995). Fas-mediated apoptosis requires clustering of the receptor in the lipid rafts (Garofalo *et al.* 2003; Grassme *et al.* 2003), which are subdomains of the plasma membrane rich in cholesterol and glycosphingolipids.

Alkyl-lysophospholipids (ALPs) such as edelfosine, perifosine, and miltefosine with potent antiproliferative and antitumor activities have shown promise in clinical trials. Edelfosine and Perifosine induce apoptosis by recruiting Fas and DISC into lipid rafts (Gajate and Mollinedo 2007). Lipid rafts serve as platform for spatially organizing specific molecules involved in signal transduction events associated with proliferation, apoptosis, cell migration, and adhesion (Galbiati *et al.* 2001). As ALPs modulate signal transduction pathways originating at the membrane level (Strassheim *et al.* 2000) and since lipid raft facilitates cross talk between different signaling pathways in glioma (Abulrob *et al.* 2004), we investigated whether miltefosine (Hexadecylphosphocholine) could induce apoptosis in GBM by altering signaling events originating at the cell membrane.

Ras proteins are members of a superfamily of related small GTPases implicated in cellular proliferation and transformation (Hunter 1997) and Ras is predominantly associated with lipid raft microdomains (Prior *et al.* 2001). Although Ras is usually associated with cell proliferation (Lloyd *et al.* 1997; Serrano *et al.* 1997); growth arrest, senescence or apoptosis in response to activated Ras has been reported (Shao *et al.* 2000). Ras not only differentially regulates discrete cell death programs through Ras- and Fas-mediated apoptotic pathways (Chen *et al.* 1998a), but it also induces non-apoptotic cell

Received June 6, 2008; revised manuscript received July 29, 2008; accepted August 4, 2008.

Address correspondence and reprint requests to Ellora Sen, National Brain Research Centre, Nainwal Mode, NH-8, Manesar, Gurgaon, Haryana-122050, India. E-mail: ellora@nbrc.res.in

Abbreviations used: ALPs, alkyl phospholipids; DISC, death-inducing signaling complex; DSB, double stranded breaks; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain; FACS, fluorescence activated cell sorter; GBM, glioblastoma multiforme; GST, glutathione-S-transferase; JNK, c-Jun N-terminal kinases; PBS, phosphate-buffered saline.

death in GBM (Chi *et al.* 1999). Proapoptotic effects of Ras are mediated by the MAPK pathway (Nesterov *et al.* 2004). The major MAPK families include extracellular signal-related kinases (ERK), c-Jun N-terminal kinases (JNK) and the p38 kinases. While ERK activation by Perifosine induces cell cycle arrest in human keratinocytes (De Siervi *et al.* 2004), Perifosine inhibits Ras/ERK signaling pathways to induce apoptosis in gliomas (Momota *et al.* 2005). Besides, ERK activation plays an active role in mediating Cisplatin-induced apoptosis of HeLa cells (Wang *et al.* 2000). Ras activation occurs in GBMs (Guha *et al.* 1996) and this high level of active Ras has been a target for Ras inhibitor mediated glioma therapy. We therefore evaluated the ability of miltefosine to affect Ras activity.

Dissecting the mechanisms of miltefosine-induced apoptosis clearly indicated that ERK activation plays a crucial role in triggering glioma cell death. Ras-induced sustained activation of ERK was concurrent with increased Fas and FADD expression. In addition, ERK-dependent increase in DNA damage repair proteins was also observed upon miltefosine treatment. Our results indicate that Ras/ERK activation plays an active role in miltefosine-induced apoptosis of glioma cells.

Materials and methods

Antibodies and inhibitors

Caveolin and p38MAPK antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and antibodies to Ras, ERK, pERK and pp38MAPK were purchased from Cell Signaling Technologies (Beverly, MA, USA). Antibody to β-actin was purchased from Sigma (St. Louis, MO, USA). Antibody to CD-71 was purchased from Zymed laboratories (San Francisco, CA, USA). Antibody to FADD was purchased from BD Biosciences (San Diego, CA, USA). H2AX and γH2AX antibodies were purchased from Upstate Biotechnology (Temecula, CA, USA). Antibody to Caspase-8 and inhibitors for ERK (U0126), p38MAPK (SB203580) and JNK (SP600125) were purchased from Calbiochem (La Jolla, CA, USA). Dominant negative Ras (Ras N17) was purchased from Clontech (Mountainview, CA, USA).

Cell culture and treatment

Glioblastoma cell lines U87MG and T98G obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. On attaining semi-confluence, cells were switched to serum-free media and after 6 h, cells were treated with different concentrations of miltefosine (Cayman Chemical, Ann Arbor, MI, USA) for different time intervals. Following treatment cells were processed for lipid raft isolation, Ras activity, Caspase activity and western blot analysis. All reagents were purchased from Sigma unless otherwise stated.

Determination of cell viability

Cell viability was assessed using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium, inner salt] (MTS) according the manufacturer's instruction Promega (Madison,WI, USA). Following treatment of cells (5×10^3) with miltefosine either in presence or absence of increasing concentration of MAPK inhibitors or 3-methyl adenine (inhibitor of autophagy), in 96-well plates, 20 μL of MTS solution was added. After 4 h of incubation the absorbance reflecting reduction of MTS by viable cells was determined at 490 nm. Values were expressed as a percentage relative to those obtained in controls. To investigate the role of Ras in miltefosine-induced death, cells were transfected with dominant negative Ras (Ras N17) carried out with Lipofectamine 2000 (Gibco-Invitrogen, Rockville, MD, USA) as described previously (Sharma *et al.* 2007). Twenty-four hours post-transfection cells were either left untreated or treated with 10 μM miltefosine for different intervals of time and cell viability determined by MTS assay.

Assay of caspases 3 and 8 activities

The Colorimetric Assay kits for caspases 3 (Sigma) and 8 (Chemicon, Temecula, CA, USA) were used to determine the enzymatic activity of caspases in glioma cells treated with miltefosine for 12 h, according to the manufacturer's instruction. The assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the substrate peptides conjugated to *p*NA. The substrate peptides used for the colorimetric assay of caspase 3 and 8 were Ac-DEVD and Ac-IETD respectively. Following treatment, cells were lysed with the lysis buffer provided and protein lysates were incubated with the colorimetric substrate with assay buffer provided at 37°C for 2 h in a 96-well microplate. The *p*NA released by cleavage of the peptide was quantified spectrophotometrically at 405 nm in a microtiter plate reader.

Western blot analysis

Thirty micrograms of protein isolated from untreated and miltefosine-treated cells was electrophoresed on 6% to 12% polyacrylamide gel and western blotting performed as described (Sharma *et al.* 2007), using the following antibodies-Fas, FADD, Caspase-8, Ras, pERK, ERK, p38MAPK, phospho-p38MAPK, γH2AX, and H2AX. Secondary antibodies were purchased from Vector Laboratories Inc. After addition of chemiluminescence reagent (Amersham, Buckinghamshire, UK) blots were exposed to Chemigenius Bioimaging System (Syngene, Cambridge, UK) for developing and images were captured using Genesnap software (Syngene). The blots were stripped and reprobed with anti-β-actin to determine equivalent loading as described (Sharma *et al.* 2007). Release of cytochrome *c* from mitochondria to cytosol was analyzed by western blot as described previously (Gajate and Mollinedo 2007), using antibody against cytochrome *c* (Santa Cruz).

Co-immunoprecipitation

For immunoprecipitation of DISC, 50–75 μg protein lysates from T98G and U87MG cells treated with miltefosine for different intervals of time were immunoprecipitated with anti-Fas antibody overnight at 4°C. The lysates were then incubated with 30 μL of protein G Plus-sepharose (Amersham) at 25°C for 2–4 h. Beads were pelleted, washed three times in immunoprecipitation buffer, and resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Western blots were performed to determine the recruitment of FADD, FAS, and Caspase 8 to the DISC.

Isolation of lipid rafts by Optiprep density centrifugation

Lipid raft isolation was performed as described previously (Stickney *et al.* 2004). Control and miltefosine-treated cells (5×10^6) were washed in phosphate-buffered saline (PBS), lysed in 267 μL of ice-cold Optibuffer [50 mM Tris (pH 7.5), 150 mM NaCl, 10% sucrose, 1 mM dithiothreitol, 1% TX-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture), transferred to microcentrifuge tubes in ice for 30 min and 533 μL of 60% Optiprep was added to the samples (to a final concentration of 40%), and the lysates were transferred to ultracentrifuge tubes. A gradient was created by the sequential layering of 800 μL of 35%, 30%, 25%, 20%, and 0% Optiprep diluted in Optibuffer on top of the samples. Samples were spun at 30 000 rpm (> 107 000 *g*) for 18 h at 4°C in a S52ST rotor (Sorvall S120ME). After centrifugation, 800-μL fractions were removed in reverse order of addition. Forty microliters of each fraction mixed with 4 μL of 10× sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer were boiled for 5 min and electrophoresed on 6% to 10% polyacrylamide gel and western blotting performed as described (Sharma *et al.* 2007), using the following antibodies – caveolin, CD-71, Fas, FADD and Ras.

Immunofluorescence microscopy

T98G cells (10^4) were treated with miltefosine in chamber slides obtained from Nunc (Roskilde, Denmark) for 12 h. Following treatment, cells were fixed in 4% paraformaldehyde for 15 min. Fixed cells were then washed twice with PBS, pre-incubated with 5% normal goat serum for 2 h at 25°C prior to incubation with mouse anti-Fas and anti-caveolin antibody overnight at 4°C. Cells were then washed thrice in PBS and incubated with anti-mouse Alexa Fluor and anti-rabbit FITC-conjugated immunoglobulins for 1 h at 25°C. After three washes with PBS, the cells were mounted with 4′-6-diamidino-2-phenylindole and the expression of Fas (red) and caveolin (green) was analyzed using a fluorescence microscope as described previously (Lacour *et al.* 2004).

Measurement of Ras activity

The Ras activity assay was performed using a commercially available Ras activation assay kit purchased from Upstate Biotechnology. Briefly, cells (2×10^6) treated with miltefosine for different time intervals, were lysed in Mg^{2+} lysis buffer. 750 μg of lysates were incubated for 1 h at 4°C with beads coated with a fusion protein [glutathione-S-transferase (GST)-Raf1-RBD] consisting of GST fused to the Ras binding domain of Raf-1. Beads were washed three times with cold Mg^{2+} containing lysis buffer, and bound protein was eluted by boiling for 5 min with 10× sample buffer and analyzed by immunoblotting for Ras.

Flow cytometric analysis of DNA content

To determine the effect of miltefosine on glioma cell cycle progression, fluorescence activated cell sorter (FACS) analysis of DNA content was performed as described (Alam *et al.* 2006). Untreated and miltefosine-treated cells were fixed in 70% ethanol, and stored at −20°C. The fixed cells were washed in PBS, resuspended in propidium iodide solution (BD Biosciences) for 20 min at 25°C and flow cytometric analysis of 10^6 cells were carried out using Cell Quest program on FACS Calibur (BD Biosciences, San Diego, CA, USA). The percentage of cells in the G1, S, and G2/M phases of the cell cycle was analyzed with the Mod Fit LT program as described (Alam *et al.* 2006).

Statistical analysis

All comparisons between groups were performed using two-tailed paired Student's *t*-test. All values of $p < 0.05$ were taken as significant.

Results

Miltefosine induces apoptosis in glioma cells

To investigate whether miltefosine affects glioma cell viability, dose-response analysis was performed where U87MG and T98G glioma cells were exposed to increasing concentrations of miltefosine for 24 h and cell viability was determined using MTS assay. Treatment with 5 μM of miltefosine for 24 h had no significant effect on glioma cell viability. A 65% decrease was observed in both U87MG and T98G upon treatment with 10 μM miltefosine, as compared with the untreated control (Fig. 1a). A further decrease in cell viability by ∼80% and 90% was observed upon treatment of T98G and U87MG respectively with 20 μM miltefosine (Fig. 1a). Miltefosine decreased viability of glioma cell lines irrespective of their p53 status (U87MG p53 wild type and T98G p53 mutant). In subsequent experiments 10 μM concentration was chosen to assess the time kinetics of cell death induced by miltefosine. Treatment with 10 μM miltefosine for 3 h had no significant effect on glioma cell viability. An ∼ 40%, 50%, and 70% decrease in viability was observed in U87MG and T98G cells at 6, 12 and 24 h post-treatment with miltefosine respectively, as compared with the control (Fig. 1b). As the ability of miltefosine to decrease viability of glioma cells by 50% reached its peak at 12 h (Fig. 1b), we treated U87MG and T98G cells with miltefosine for 12 h to dissect its mechanisms of action in all subsequent experiments.

Increased cell death was accompanied by a significant ∼2.2 and 3.7-fold increase in active caspase-3 levels in U87MG treated with miltefosine for 6 and 12 h respectively, as compared with control (Fig. 1c). Similar trend in Caspase 3 activity was observed in miltefosine-treated T98G cells (Fig. 1c). Since the activation of caspase-3-like proteases is crucial in apoptotic cell death (Kumar and Lavin 1996), these results suggest that miltefosine induce apoptosis in glioma cells.

Induction of glioma cell apoptosis by miltefosine involves formation of death inducing signaling complex

The Fas receptor/ligand system has been implicated to be involved in miltefosine-induced apoptosis (Cabaner *et al.* 1999). Fas interacts with FADD to recruit procaspase-8 resulting in the formation of DISC (Kischkel *et al.* 1995) and activation of caspase-8 further activates caspase-3 that

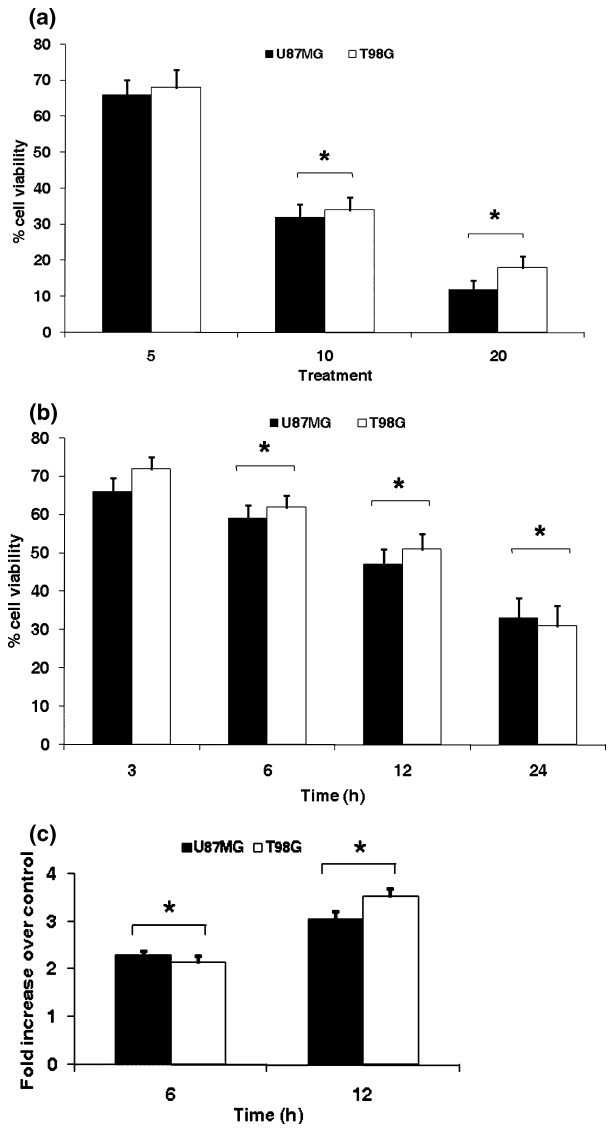

Fig. 1 Miltefosine induces apoptosis in glioma cells (a) Miltefosine decreases viability of glioma cells in a dose-dependent manner. U87MG and T98G cells (5×10^3) were treated with 5–20 μM miltefosine for 24 h, and cells were subjected to MTS assay. A decrease in glioma cell proliferation upon treatment with increasing concentration of miltefosine was observed. (b) A time kinetics depicting decrease in glioma cell viability with increasing exposure to miltefosine. U87MG and T98G cells (5×10^3) were treated with 10 μM miltefosine for different time intervals and cells were subjected to MTS assay. (c) The levels of active caspase-3 in U87MG and T98G cells treated with miltefosine for 6 and 12 h, as determined by the Caspase-3 activity assay. Values in (a), (b) and (c) represent the means ± SEM from three independent experiments. *Significant decrease from control ($p < 0.05$).

initiates apoptosis. Western blot analysis revealed a gradual increase in Fas, FADD and caspase 8 levels in U87MG and T98G cells upon increasing exposure to miltefosine (Fig. 2a).

Fas has the ability to interact with FADD directly which recruits procaspase 8 to form DISC (Kischkel *et al.* 1995). Since Fas expression was increased in glioma cells upon exposure to miltefosine, co-immunoprecipitation experiments were performed to investigate whether miltefosine induced the formation of DISC, involving death receptors and death-linked adapter proteins. Lysates from untreated and miltefosine-treated glioma cells were immunoprecipitated with anti-Fas antibody, followed by western blotting either with anti-FADD or anti-caspase 8. FADD and cleaved caspase 8 were detected in immunoprecipitates in miltefosine-treated U87MG and T98G but not from control (Fig. 2b). While treatment of glioma cells with miltefosine for 3 h had no significant effect on the association of Fas and FADD with caspase-8, treatment for 6 and 12 h significantly increased DISC formation, as compared with the control (Fig. 2b).

Since increase in active caspase-8 level was observed in the DISC from miltefosine-treated cells, we determined the levels of caspase-8 activity in these cells. A 2.5 and 5-fold increase in caspase-8 activity was observed in both U87MG and T98G cells treated with miltefosine for 6 and 12 h respectively, as compared with the control (Fig. 2c). Thus, treatment of glioma cells with miltefosine significantly elevates caspase-8 activity.

Miltefosine increases clustering of Fas towards the lipid raft microdomain

As membrane lipid rafts play critical role in Fas-mediated cell death (Garofalo *et al.* 2003; Grassme *et al.* 2003; Lacour *et al.* 2004), we investigated whether miltefosine treatment affects the localization of Fas in the lipid raft microdomains as reported with other ALPs (Gajate and Mollinedo 2007). Lysates from untreated and miltefosine-treated cells were subjected to centrifugation on Optiprep density gradient and each fraction from this density gradient was analyzed using immunoblots for the presence of raft marker caveolin and non-raft marker CD71 (Fig. 3a). In untreated cells Fas and FADD was mostly localized in the non-raft fractions positive for CD71 and less in the caveolin rich fractions, suggesting that Fas and FADD is expressed predominantly in the non-raft fraction in T98G and U87MG glioma cells (Fig. 3a). However, treatment with miltefosine increased the distribution of Fas more towards the raft fraction (Fig. 3a). The increased clustering of Fas into raft fraction was accompanied by their increased expression (Fig. 3a). Although treatment with miltefosine did not effect the distribution of FADD towards the raft domain as much as Fas, the increase in FADD expression in miltefosine-treated cells was clearly evident (Fig. 3a).

As co-clustering of Fas in lipid rafts is a crucial event in the regulation of apoptosis by anticancer drugs (Gajate and Mollinedo 2005), we investigated the localization of caveolin with Fas in miltefosine-treated cells. Fluorescence microscopic analysis revealed an intense co-localization of Fas with caveolin in miltefosine-treated T98G cells. This is in

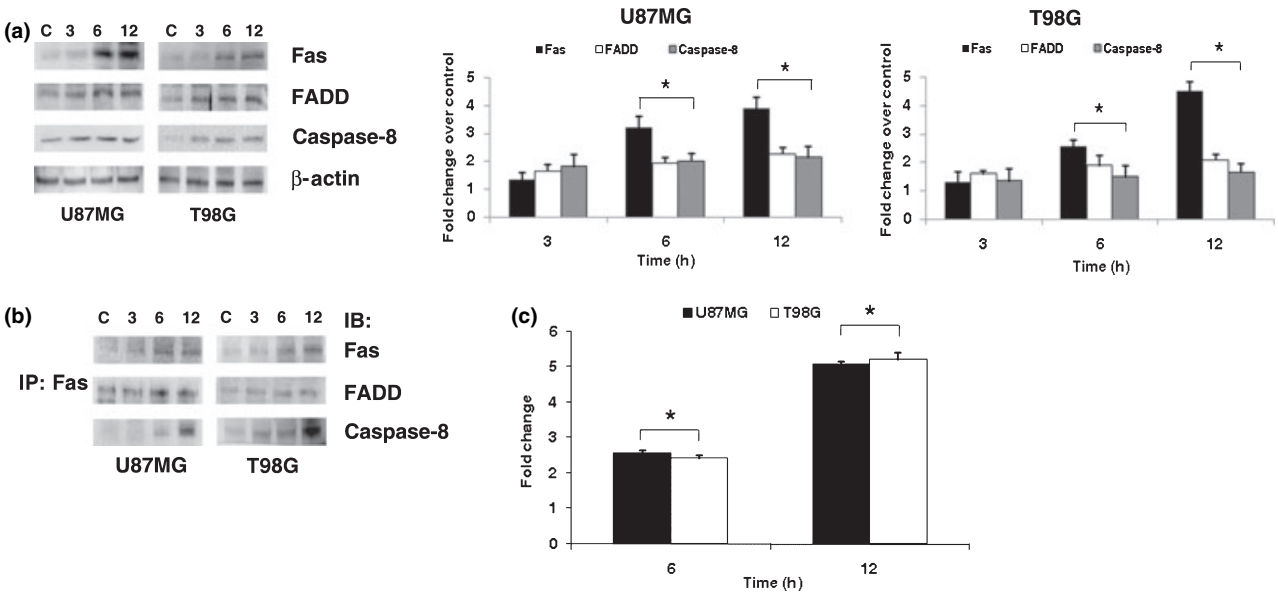

Fig. 2 Miltefosine sensitizes glioma cells to Fas-mediated apoptosis by inducing the formation of Death Inducing Signaling Complex (DISC). (a) Miltefosine induces Fas, FADD and cleaved caspase-8 expressions in glioma cells. Western blot analysis revealed an increase in Fas, FADD and cleaved caspase-8 levels in U87MG and T98G cells upon increasing exposure to 10 μM miltefosine. A representative blot is shown from three independent experiments with identical results. Blots were reprobed for β-actin to establish equivalent loading. Densitometric measurements were performed on individual immunoblots for each antibody tested and values represent the means ± SEM from three individual experiments. *Significant change from control ($p < 0.05$). (b) Miltefosine treatment induces DISC formation in glioma cells. Cell lysate from control and miltefosine-treated U87MG and T98G cells were immunoprecipitated with antibody to Fas and DISC components – Fas, FADD and cleaved caspase-8 were analyzed by immunoblotting. A representative blot is shown from two independent experiments with identical results. (c) Fold increase in caspase-8 activity in glioma cells treated with miltefosine for 6 and 12 h, as determined by the Caspase-8 activity assay. Values represent the mean ± SEM from three independent experiments. * Significant change from control ($p < 0.05$).

striking contrast to untreated cells, where the co-localization of Fas and caveolin is almost negligible and less intense. These observations further confirmed that miltefosine increases the distribution of Fas towards the raft microdomains (Fig. 3b).

The disappearance of cytochrome *c* from the mitochondrial fraction in ALP-treated cells by the time Fas is translocated into lipid rafts (Gajate and Mollinedo 2007), have been reported. To investigate the involvement of mitochondria in miltefosine-induced glioma cell apoptosis, cytochrome *c* level in mitochondria and cytosol was determined in miltefosine-treated cells. Cytosolic cytochrome *c* level was increased and its expression in mitochondria was decreased following treatment with miltefosine (Fig. 3c). This increased translocation of mitochondrial cytochrome *c* into the cytosol suggested mitochondrial involvement in miltefosine-induced cell death.

Miltefosine increases both the expression and activity of Ras in glioma cells

As oncogenic Ras induces apoptosis through Fas-mediated signaling (Chen *et al.* 1998a) and since miltefosine triggered apoptosis through Fas-mediated DISC formation in glioma cells, we investigated the contribution of Ras in miltefosine-induced Fas-mediated apoptosis. Western blot analysis demonstrated an increase in Ras expression in glioma cells upon increasing exposure to miltefosine (Fig. 4a).

As Ras levels were elevated in miltefosine-treated cells, we investigated the effect of miltefosine on Ras activity. Lysates from cells grown in the presence or the absence of miltefosine for different intervals of time were subjected to a Ras-GTP pull-down assay (de Rooij and Bos 1997). Ras-GTP was detectable in untreated gliomas and the levels of activated Ras were dramatically elevated in glioma cells upon miltefosine treatment (Fig. 4b). Not only was Ras activity elevated at 1 h following miltefosine treatment, this heightened level of activated Ras was sustained even at 12 h post-treatment (Fig. 4b).

Miltefosine does not affect Ras localization in lipid raft microdomain

Ras is predominantly associated with lipid raft microdomains (Prior *et al.* 2001). As miltefosine increased the clustering of Fas and FADD into lipid raft domain, we investigated whether increase in Ras levels was accompanied by altered distribution of Ras between rafts and non-raft portion

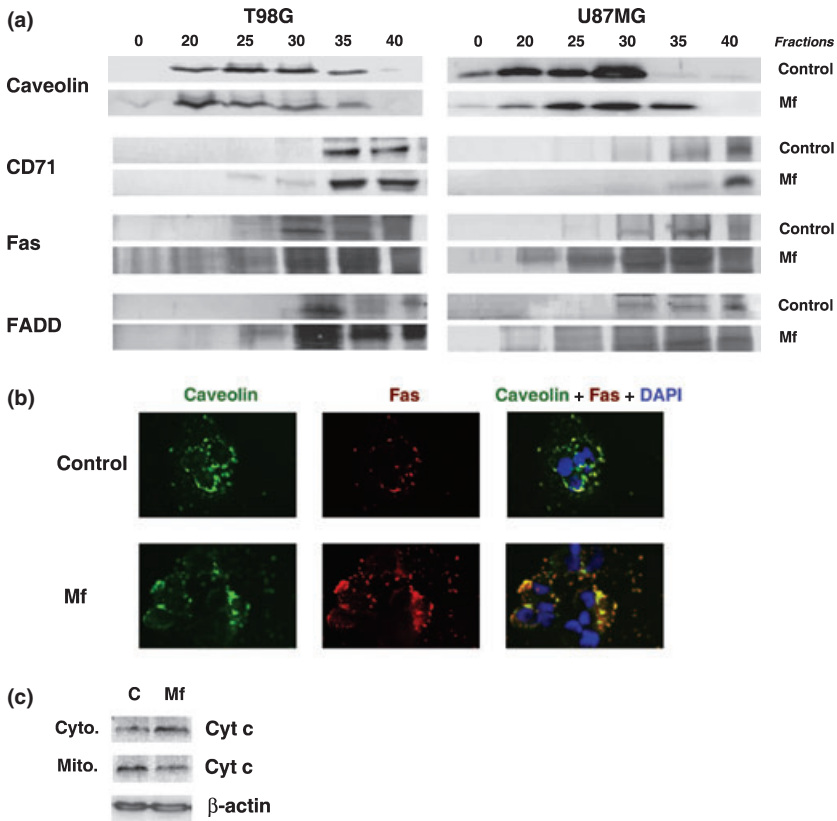

Fig. 3 Miltefosine increases the clustering of Fas towards lipid raft microdomains of glioma cells (a) Raft and non-raft fractions were isolated from control and miltefosine-treated U87MG and T98G cell lysates by optiprep gradient centrifugation. The fractions were subjected to western blot analysis to determine the localization of raft marker caveolin-1 and non-raft marker CD71. Fas and FADD mostly co-localized with the non-raft fraction in control cells and treatment with miltefosine increased their distribution in the raft portion. A representative blot is shown from three independent experiments with identical results. (b) Miltefosine increases Fas co-localization with caveolin as demonstrated by fluorescence microscopy. T98G cells were left untreated or treated with miltefosine for 12 h, followed by incubation with antibodies against Fas and caveolin. Cells were then washed and labeled by incubating with Alexa Fluor (Red, Fas) and FITC (Green, caveolin). Increased co-localization between caveolin and Fas in miltefosine-treated cells was observed in the merge panel (yellow). One representative image of three independent experiments is shown. (c) Involvement of mitochondria in miltefosine-induced glioma cell death. Western blot analysis of cytochrome *c* in cytosolic and mitochondrial extracts from T98G glioma cells treated in the presence or absence of miltefosine for 12 h. A representative blot is shown from two independent experiments with identical results.

(Goodwin *et al.* 2005). Western blot analysis revealed that Ras is localized in the raft portion of T98G and U87MG cells and it accumulates further upon miltefosine treatment (Fig. 4b). However, unlike Fas localization, treatment with miltefosine had no effect on the distribution of Ras, which remained confined to raft microdomain even after exposure to miltefosine for 12 h (Fig. 4c).

Increased Ras activity in miltefosine-treated glioma cells does not induce autophagy

In GBM oncogenic Ras-induced cell death has been shown to be consistent with autophagy (Chi *et al.* 1999). As miltefosine elevated Ras activity in glioma cells, we determined whether miltefosine induces autophagy in GBM, in addition to apoptosis. Treatment of glioma cells with autophagy inhibitor 3-methyladenine (3-MA) had no significant effect on glioma cell viability. Co-treatment of U87MG and T98G cells with Milefosine and 3-MA had no effect on the cytotoxic effect of miltefosine (Fig. 4d), as the viability of miltefosine-treated cells observed in the presence and absence of 3-MA was comparable. Together with the caspase3 and 8 activity results, this suggests that miltefosine-induced glioma cell death is apoptotic and not autophagic.

Involvement of Ras activity in miltefosine-induced cell death

To investigate the role of elevated Ras activity in miltefosine-induced cell death, glioma cells transfected with dominant-negative Ras (RasN17) were treated with miltefosine. Expression of RasN17 abrogated miltefosine-induced cell death significantly (Fig. 5a). While a 20% increase in cell viability was observed in Ras N17 transfected cells treated

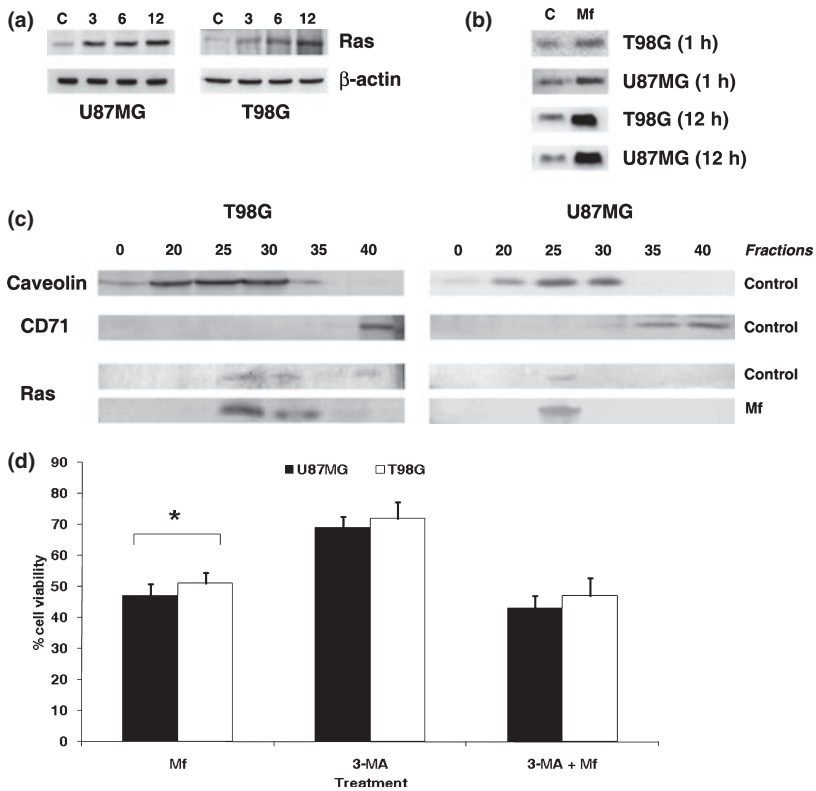

Fig. 4 Miltefosine-induced increase in Ras expression and activity has no effect on its localization on raft domain nor does it induce autophagy in glioma cells. (a) Miltefosine increases Ras expression in glioma cells in a time-dependent manner. U87MG and T98G cells were treated with 10 μM miltefosine for different intervals of time and Ras levels were determined by western blot analysis. A representative blot is shown from three independent experiments with identical results. Blots were reprobed for β-actin to establish equivalent loading. (b) Effects of miltefosine on the levels of GTP-bound Ras. The levels of Ras-GTP in protein extracts of control and miltefosine (10 μM)-treated U87MG and T98G cells were determined by the ability of Ras-GTP to bind to a specific protein domain of Raf in the form of a GST-fusion protein. An increase in Ras activity was observed in U87MG and T98G cells treated with miltefosine for different time intervals, as compared to the control. The figure is representative from four independent experiments with identical results. Mf denotes miltefosine. (c) The localization of Ras in raft microdomains in glioma cells is unaffected by miltefosine treatment. The distribution of Ras in raft and non-raft portion of control and miltefosine-treated U87MG and T98G cells was determined as described in legends to Fig. 3. While Ras localization remained unaffected upon miltefosine treatment, an increase in its expression was observed. (d) Miltefosine treatment does not induce autophagy in glioma cells. U87MG and T98G cells were treated with 10 μM miltefosine in the presence or absence of 10 mM 3-MA (inhibitor of autophagy) for 12 h, and cell viability was determined by MTS assay. The graph represents the percentage of viable cells as determined by MTS assay, observed when glioma cells were treated with either miltefosine, or 3-MA or both for 12 h. The viability of miltefosine-treated cells observed in the presence and absence of 3-MA was comparable. Values represent the means ± SEM from three independent experiments. *Significant increase from control ($p < 0.05$).

with miltefosine for 3 h, a 30% increase in viability was observed at 6 and 12 h post-treatment as compared with the cells treated with miltefosine alone. The inhibition of miltefosine-induced cell death in RasN17 transfected cells indicated that proapoptotic effects of miltefosine is Ras-dependent.

Inhibition of ERK and p38MAPK activation decreases the proapoptotic effects of miltefosine

Increased Ras activity induces apoptosis (Chen *et al.* 1998a) and pro-apoptotic effects of Ras are mediated by the MAPK pathway (Nesterov *et al.* 2004). ERK activation plays an active role in mediating Cisplatin-induced apoptosis of HeLa cells (Wang *et al.* 2000), and Perifosine is known to activate MAPK pathways (De Siervi *et al.* 2004). Since miltefosine-mediated apoptosis was concomitant with elevated Ras activity, we determined whether altered MAPK pathway signaling was involved in miltefosine-induced cell death. The major MAPK families include ERK, JNK, and p38MAPK. Inhibitor U0126, SB203580, and SP600125 inhibit responses mediated by ERK, p38MAPK, and JNK signaling, respectively. We next determined the viability of glioma cells treated with miltefosine in the presence and in the absence of ERK, JNK, and p38MAPK inhibitors, to confirm the role of

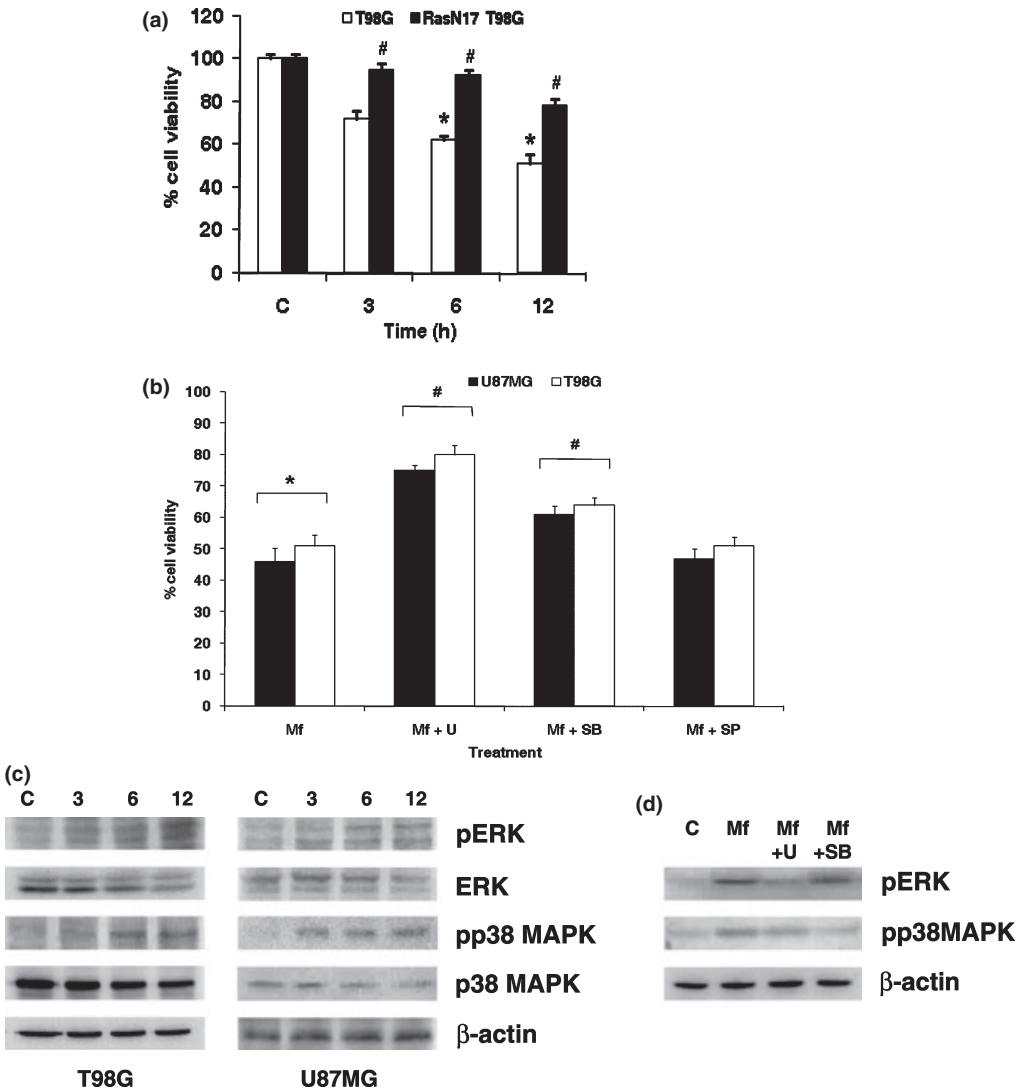

Fig. 5 The pro-apoptotic effect of Mitefosine is mediated by Ras and ERK/p38MAPK activation. (a) Increased Ras activity is involved in miltefosine-mediated cell death. Untransfected and dominant negative Ras (Ras N17) transfected T98G cells were treated with 10 μM miltefosine for different intervals of time and cell viability was determined by MTS assay. The graph represents the percentage of viable cells as determined by MTS assay, observed when untransfected or RasN17 transfected glioma cells were treated with miltefosine. RasN17 transfected cells had greater viability in the presence of miltefosine as compared to cells treated with miltefosine alone. Values represent the means ± SEM from three independent experiments. *Significant decrease from control ($p < 0.05$). #Significant increase from miltefosine-treated cells ($p < 0.05$). (b) Miltefosine-induced cell death is decreased in the presence of ERK and to a lesser extent by p38MAPK inhibitor. The graph represents the percentage of viable cells as determined by MTS assay, observed when U87MG and T98G cells were treated with miltefosine in the presence or absence of ERK, p38MAPK and JNK inhibitors. Cells were treated with 10 μM miltefosine either alone or in combination with either 20 μM U0126 or 20 μM SB203580 or 10 μM SP600125 for 12 h. Values in (a) and (b) represents the mean ± SEM from three independent experiments. *Significant change from control. #Significant change from miltefosine-treated cells ($p < 0.05$). (c) Miltefosine increases ERK and p38MAPK phosphorylation in glioma cells. Western blot analysis was performed to detect the expression of ERK, pERK, p38MAPK and phospho-p38MAPK in glioma cells treated with 10 μM miltefosine for different intervals of time. An increase in ERK and p38MAPK phosphorylation and decrease in total ERK and p38MAPK level was observed in glioma cells upon increasing exposure to miltefosine, as compared to the untreated control. (d) Selective inhibition of miltefosine-induced pERK and p38MAPK activity in the presence of their respective inhibitors. Western blot analysis was performed to detect whether the elevated pERK and phospho-p38MAPK in miltefosine-treated glioma cells could be inhibited in the presence of ERK (U0126) and p38MAPK (SB203580) inhibitor, respectively. The increase in pERK and pp38MAPK observed miltefosine-treated cells was decreased in the presence of U0126 and SB203580, respectively. (c and d) representative blots are shown from three independent experiments with identical results. Blots were reprobed for β-actin to establish equivalent loading.

these pathways in miltefosine-induced cell death. The concentration of ERK, JNK, and p38MAPK inhibitors used were chosen on the basis of dose kinetics. The effect of these inhibitors were dose-dependent saturating at a higher concentration (Fig. S1 in Supporting information). Although treatment of glioma cells with ERK or p38MAPK inhibitor alone had no significant effect on cell viability, treatment with JNK inhibitor alone at higher concentration induced considerable cell death (Fig. S1 in Supporting information). An approximately 25 and 30% increase in cell viability was observed in U87MG and T98G respectively, when treated with a combination of miltefosine and ERK inhibitor U0126, as compared with that treated with miltefosine alone (Fig. 5b). On the other hand, co-treatment with miltefosine and p38MAPK inhibitor SB203580 resulted in a marginal (~12%) but significant increase in cell viability, as compared with cells treated with miltefosine alone. However, treatment with JNK inhibitor had no effect on miltefosine-induced cell death, as the viability of miltefosine-treated cells observed in the presence and in the absence of JNK inhibitor SP600125 was comparable (Fig.5b). These results indicate that ERK and to a lesser extent p38MAPK activation plays a major role in miltefosine-induced apoptosis.

We next chose to investigate the role of ERK and p38MAPK in miltefosine-induced glioma cell death by determining the status of ERK and p38MAPK activation upon miltefosine treatment. An elevation in ERK and p38MAPK phosphorylation was observed in glioma cells upon increasing exposure to miltefosine, as compared with the untreated control (Fig. 5c).

To further confirm the involvement of ERK and p38MAPK in miltefosine-induced apoptosis, we determined the specificity of the inhibitors. The increase in pERK and p38MAPK observed in the presence of miltefosine was decreased considerably in the presence of their respective inhibitors (Fig. 5d). On the other hand, no noticeable change in pERK or p38MAPK was observed in the presence of SB203580 or U0126, respectively. This selective inhibition of pERK and p38MAPK in miltefosine-treated cells upon treatment with specific inhibitors further confirmed the involvement of these two pathways in miltefosine-induced glioma cell apoptosis, although to different extent (Fig. 5d).

Miltefosine-induced Fas and FADD expression are ERK-dependent

Ras-mediated apoptosis is FADD and caspase 8-dependent (Chen *et al.* 2001). The dramatic decrease in miltefosine-induced cell death in the presence of ERK inhibitor and to a lesser extent with p38MAPK inhibitor, suggested the importance of MAPK activation in miltefosine induced glioma cell death. To determine whether ERK and p38MAPK pathway modulate Fas triggered apoptosis, we determined the expression of Fas and FADD in glioma cells treated with miltefosine in the presence of ERK and p38MAPK inhibitors. The increased expression of Fas and FADD observed upon miltefosine treatment was down-regulated in the presence of ERK but not p38MAPK inhibitor (Fig. 6). This finding suggests the greater involvement of ERK and not p38MAPK pathway in triggering Fas-mediated apoptosis in miltefosine-treated cells.

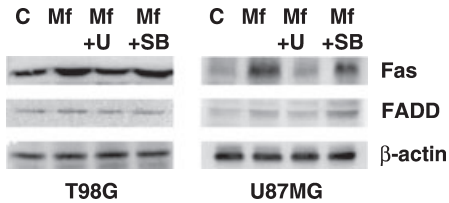

Fig. 6 Inhibition of ERK activation regulates miltefosine-induced Fas and FADD expression in glioma cells. U87MG and T98G cells were treated with 10 μM miltefosine in the presence or absence of 20 μM U0126 or 20 μM SB203580 and western blot analysis was performed. The increase in Fas and FADD expression observed in miltefosine-treated cells was down-regulated in the presence of ERK but not p38MAPK inhibitors. Representative blot is shown from three independent experiments with identical results. Blots were reprobed for β-actin to establish equivalent loading.

Miltefosine induces glioma cell cycle arrest

Since cell cycle arrest is necessary for Ras-mediated apoptosis (Liou *et al.* 2000) and as ALP Perifosine induces squamous carcinoma cell cycle arrest (Patel *et al.* 2002), we performed FACS analysis to study the effect of miltefosine on glioma cell cycle (Fig. 7a). An increase in the percentage of cells at G2/M phase was observed in T98G cells at 12 h post-treatment with miltefosine, as compared with the untreated control (Fig. 7a). Similar trend was observed in U87MG cells (data not shown). Our results agree with previous observation that ALPs induce cell cycle arrest.

Miltefosine-induced increase in DNA DSB repair proteins is ERK regulated

Ras induces DNA damage signaling response (Weidhaas *et al.* 2006) and cells that senesce in response to oncogenic Ras accumulate DNA damage foci (Mallette *et al.* 2007). Histone H2AX is rapidly phosphorylated surrounding a DNA double-strand break and this phosphorylated H2AX (γ-H2AX) is involved in the recruitment of DNA repair factors at sites of double stranded breaks (DSBs). Since increased Ras/ERK activation was observed in miltefosine-treated glioma cells, we determined the expression of γ-H2AX in T98G cells treated with miltefosine in the presence and absence of ERK and p38MAPK inhibitors. The level of γ-H2AX was elevated in miltefosine-treated cells, as compared with the untreated control (Fig.7b). While the elevated levels of γH2AX were abrogated to control levels in the presence of ERK inhibitor, γH2AX level in miltefosine-treated cells was not affected in the presence of p38MAPK inhibitor (Fig. 7b). Similar trend was observed in the

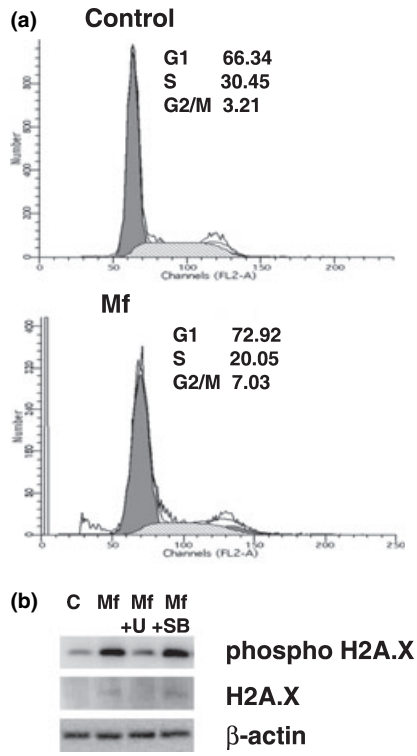

Fig. 7 Miltefosine induces cell cycle arrest and increases DNA damage signaling response (a) Treatment of glioma cells with miltefosine increases accumulation of cells in G2/M phase of the cell cycle. FACS analysis was performed on cells treated with 10 μM miltefosine and the percentages of cells in the various cell cycle phases were plotted. FACS analysis profile of miltefosine treated and control is shown. Insets indicated percentage cells in G1, S and G2/M phase of the cell cycle in U87MG cells. C and Mf denote control and miltefosine respectively. (b) Miltefosine increases accumulation of proteins involved in the repair of DNA DSB. Ability of glioma cells to repair DNA damage response in the presence and absence of ERK and p38MAPK inhibitors were detected by determining the expression of γ-H2AX. Protein isolated from glioma cells treated with 10 μM miltefosine for 12 h in the presence or absence of 20 μM U0126 or 20 μM SB203580 and western blot analysis was performed to detect the expression of phosphorylation of H2AX (Ser^{139}) and total H2AX. Increased γ-H2AX and H2AX levels observed in miltefosine-treated glioma cells was abrogated in the presence of ERK but not p38MAPK inhibitor. The blot shown is a representative from two independent experiments with identical results. Blots were reprobed for β-actin to establish equivalent loading.

expression of total H2AX (Fig. 7b). These results indicate the involvement of ERK in miltefosine-induced accumulation of γH2AX in glioma cells.

Discussion

Despite Fas being expressed in majority of GBM (Tachibana *et al.* 1995), most glioma cells are resistant to Fas-mediated apoptosis (Riffkin *et al.* 2001). Lipid rafts play a crucial role in the signal transduction of Fas, since its translocation into raft microdomains triggers the chain of events leading to caspase-3 activation (Mandal *et al.* 2005). Moreover, Fas aggregation and recruitment of apoptotic molecules in Fas-enriched raft microdomains by ALPs, has been suggested as an effective mechanism of triggering Fas-mediated apoptosis in tumor cells (Gajate *et al.* 2004). It is possible that miltefosine treatment facilitates the concentration of DISC in Fas-enriched raft microdomains, to trigger the switch in signaling pathway leading to apoptosis.

In contrast to various other tumors, mutation of proto-oncogene Ras which constitutively activates Ras protein is rare in GBM (Bos 1989). Although GBM do not harbor oncogenic Ras mutations (Bos 1989); Ras activation occurs in GBMs (Guha *et al.* 1996). As a consequence, this high level of active Ras has been a target for Ras inhibitors mediated glioma therapy (Goldberg and Kloog 2006). However, cellular response to activated Ras depend on the cell type, with Ras either inhibiting or promoting apoptosis depending on the presence of other pro-apoptotic or anti-apoptotic signals (Downward 1998). Ras not only regulates discrete apoptotic pathways (Chen *et al.* 1998a) but can also trigger caspase-independent cell death in human cancer cells (Chi *et al.* 1999). Although the ALP Perifosine induces apoptosis in glioma cells by inactivating Ras/ERK pathways (Momota *et al.* 2005), our findings agree with previous studies that suggest the importance of elevated ERK activation in induction of apoptosis (Wang *et al.* 2000).

Our studies demonstrated that miltefosine is capable of inducing apoptosis in glioma cell lines, and the induction is mediated by Fas and increased Ras activity. These findings are opposite to the common observations that increased Ras activity in glioma cells is responsible for tumorgenicity and inhibition of Ras activity leads to growth inhibition and cell cycle arrest. The involvement of Ras as a modulator of Fas-mediated apoptosis is well established (Gulbins *et al.* 1995; Chen *et al.* 2001) with Ras-mediated apoptosis being FADD and caspase 8-dependent (Chen *et al.* 2001). It was not surprising that miltefosine-mediated increase in Ras activity was accompanied with increased sensitivity of glioma cells to Fas-mediated apoptosis. Despite the increase in both ERK and p38MAPK phosphorylation in miltefosine-treated cells, it was ERK activation that decreased the pro-apoptotic effect of miltefosine considerably through Fas regulation. The ability of enhanced Ras/ERK activity to amplify the intensity of miltefosine-mediated apoptosis by increasing Fas and FADD expression, is in line with the findings that MEK activation enhances DISC in Ras-transformed cells (Nesterov *et al.* 2004). These findings warrant attention; given that the current strategies in anti-glioma therapeutics are aimed towards inhibiting the increased Ras activity.

The induction of DSBs triggers a number of protective responses including the enhancement of repair pathways,

initiation of cell cycle arrest and the induction of cell death. The ERK-dependent increase in γH2AX in miltefosine-treated glioma cells suggests the involvement of ERK in facilitating the local concentration of repair-related proteins within the vicinity of DNA DSBs. It is known that genomic instability renders cells with activated Ras more susceptible to apoptosis, possibly through the accumulation of undesirable or lethal genetic events (Chen *et al.* 1998b). Also, the cyclin-dependent kinase inhibitor Flavopiridol has been reported to enhance human tumor cell radiosensitivity by increasing prolonged γH2AX expression (Russell *et al.* 2003). The increased γH2AX accumulation coupled with the ability of Mitefosine to induce glioma cell apoptosis independent of the cellular p53 status, may be a promising therapeutic approach in GBM given the fact that GBM are resistant to pro-apoptotic therapeutics.

Acknowledgments

This work was supported by the Innovative Young Biotechnologist Award (IYBA) research grant from the Department of Biotechnology, Government of India, to ES.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Dose-dependent kinetics depicting the specificity of MAPK inhibitors on miltefosine-induced cell death.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting material supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

Abulrob A., Giuseppin S., Andrade M. F., McDermid A., Moreno M. and Stanimirovic D. (2004) Interactions of EGFR and caveolin-1 in human glioblastoma cells: evidence that tyrosine phosphorylation regulates EGFR association with caveolae. *Oncogene* **23**, 6967–6979.

Alam S., Sen E., Brashear H. and Meyers C. (2006) Adeno-associated virus type 2 increases proteosome-dependent degradation of p21WAF1 in a human papillomavirus type 31b-positive cervical carcinoma line. *J. Virol.* **80**, 4927–4939.

Bos J. L. (1989) ras oncogenes in human cancer: a review. *Cancer Res.* **49**, 4682–4689.

Cabaner C., Gajate C., Macho A., Munoz E., Modolell M. and Mollinedo F. (1999) Induction of apoptosis in human mitogen-activated peripheral blood T-lymphocytes by the ether phospholipid ET-18-OCH3: involvement of the Fas receptor/ligand system. *Br. J. Pharmacol.* **127**, 813–825.

Chen C. Y., Liou J., Forman L. W. and Faller D. V. (1998a) Differential regulation of discrete apoptotic pathways by Ras. *J. Biol. Chem.* **273**, 16700–16709.

Chen C. Y., Liou J., Forman L. W. and Faller D. V. (1998b) Correlation of genetic instability and apoptosis in the presence of oncogenic Ki-Ras. *Cell Death Differ.* **5**, 984–995.

Chen C. Y., Juo P., Liou J. S., Li C. Q., Yu Q., Blenis J. and Faller D. V. (2001) The recruitment of Fas-associated death domain/caspase-8 in Ras-induced apoptosis. *Cell Growth Differ.* **12**, 297–306.

Chi S., Kitanaka C., Noguchi K. *et al.* (1999) Oncogenic Ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. *Oncogene* **18**, 2281–2290.

De Siervi A., Marinissen M., Diggs J., Wang X. F., Pages G. and Senderowicz A. (2004) Transcriptional activation of p21(waf1/cip1) by alkylphospholipids: role of the mitogen-activated protein kinase pathway in the transactivation of the human p21(waf1/cip1) promoter by Sp1. *Cancer Res.* **64**, 743–750.

DeAngelis L. M. (2001) Brain tumors. *N. Engl. J. Med.* **344**, 114–123.

Downward J. (1998) Ras signalling and apoptosis. *Curr. Opin. Genet. Dev.* **8**, 49–54.

Gajate C. and Mollinedo F. (2005) Cytoskeleton-mediated death receptor and ligand concentration in lipid rafts forms apoptosis-promoting clusters in cancer chemotherapy. *J.Biol. Chem.* **280**, 11641–11647.

Gajate C. and Mollinedo F. (2007) Edelfosine and perifosine induce selective apoptosis in multiple myeloma by recruitment of death receptors and downstream signaling molecules into lipid rafts. *Blood* **109**, 711–719.

Gajate C., Del Canto-Janez E., Acuna A. U., Amat-Guerri F., Geijo E., Santos-Beneit A. M., Veldman R. J. and Mollinedo F. (2004) Intracellular triggering of Fas aggregation and recruitment of apoptotic molecules into Fas-enriched rafts in selective tumor cell apoptosis. *J. Exp. Med.* **200**, 353–365.

Galbiati F., Razani B. and Lisanti M. P. (2001) Emerging themes in lipid rafts and caveolae. *Cell* **106**, 403–411.

Garofalo T., Misasi R., Mattei V., Giammarioli A. M., Malorni W., Pontieri G. M., Pavan A. and Sorice M. (2003) Association of the death-inducing signaling complex with microdomains after triggering through CD95/Fas. Evidence for caspase-8-ganglioside interaction in T cells. *J. Biol. Chem.* **278**, 8309–8315.

Goldberg L. and Kloog Y. (2006) A Ras inhibitor tilts the balance between Rac and Rho and blocks phosphatidylinositol 3-kinase-dependent glioblastoma cell migration. *Cancer Res.* **66**, 11709–11717.

Goodwin J. S., Drake K. R., Remmert C. L. and Kenworthy A. K. (2005) Ras diffusion is sensitive to plasma membrane viscosity. *Biophys. J.* **89**, 1398–1410.

Grassme H., Cremesti A., Kolesnick R. and Gulbins E. (2003) Ceramide-mediated clustering is required for CD95-DISC formation. *Oncogene* **22**, 5457–5470.

Guha A., Lau N., Huvar I., Gutmann D., Provias J., Pawson T. and Boss G. (1996) Ras-GTP levels are elevated in human NF1 peripheral nerve tumors. *Oncogene* **12**, 507–513.

Gulbins E., Bissonnette R., Mahboubi A. *et al.* (1995) FAS-induced apoptosis is mediated via a ceramide-initiated RAS signaling pathway. *Immunity* **2**, 341–351.

Hunter T. (1997) Oncoprotein networks. *Cell* **88**, 333–346.

Kischkel F. C., Hellbardt S., Behrmann I., Germer M., Pawlita M., Krammer P. H. and Peter M. E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J.* **14**, 5579–5588.

Kumar S. and Lavin M. F. (1996) The ICE family of cysteine proteases as effectors of cell death. *Cell Death Differ.* **3**, 255–267.

Lacour S., Hammann A., Grazide S., Lagadic-Gossmann D., Athias A., Sergent O., Laurent G., Gambert P., Solary E. and Dimanche-Boitrel M. T. (2004) Cisplatin-induced CD95 redistribution into membrane lipid rafts of HT29 human colon cancer cells. *Cancer Res.* **64**, 3593–3598.

Liou J. S., Chen C. Y., Chen J. S. and Faller D. V. (2000) Oncogenic ras mediates apoptosis in response to protein kinase C inhibition

through the generation of reactive oxygen species. *J. Biol. Chem.* **275**, 39001–39011.

Lloyd A. C., Obermuller F., Staddon S., Barth C. F., McMahon M. and Land H. (1997) Cooperating oncogenes converge to regulate cyclin/cdk complexes. *Genes Dev.* **11**, 663–677.

Mallette F. A., Gaumont-Leclerc M. F. and Ferbeyre G. (2007) The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev.* **21**, 43–48.

Mandal D., Mazumder A., Das P., Kundu M. and Basu J. (2005) Fas-, caspase 8-, and caspase 3-dependent signaling regulates the activity of the aminophospholipid translocase and phosphatidylserine externalization in human erythrocytes. *J. Biol. Chem.* **280**, 39460–39467.

Momota H., Nerio E. and Holland E. C. (2005) Perifosine inhibits multiple signaling pathways in glial progenitors and cooperates with temozolomide to arrest cell proliferation in gliomas in vivo. *Cancer Res.* **65**, 7429–7435.

Nesterov A., Nikrad M., Johnson T. and Kraft A. S. (2004) Oncogenic Ras sensitizes normal human cells to tumor necrosis factor-alpha-related apoptosis-inducing ligand-induced apoptosis. *Cancer Res.* **64**, 3922–3927.

Patel V., Lahusen T., Sy T., Sausville E. A., Gutkind J. S. and Senderowicz A. M. (2002) Perifosine, a novel alkylphospholipid, induces p21(WAF1) expression in squamous carcinoma cells through a p53-independent pathway, leading to loss in cyclin-dependent kinase activity and cell cycle arrest. *Cancer Res.* **62**, 1401–1409.

Prior I. A., Harding A., Yan J., Sluimer J., Parton R. G. and Hancock J. F. (2001) GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. *Nat. Cell Biol.* **3**, 368–375.

Riffkin C. D., Gray A. Z., Hawkins C. J., Chow C. W. and Ashley D. M. (2001) Ex vivo pediatric brain tumors express Fas (CD95) and FasL (CD95L) and are resistant to apoptosis induction. *Neurooncology* **3**, 229–240.

de Rooij J. and Bos J. L. (1997) Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. *Oncogene* **14**, 623–625.

Russell J. S., Brady K., Burgan W. E., Cerra M. A., Oswald K. A., Camphausen K. and Tofilon P. J. (2003) Gleevec-mediated inhibition of Rad51 expression and enhancement of tumor cell radiosensitivity. *Cancer Res.* **63**, 7377–7383.

Serrano M., Lin A. W., McCurrach M. E., Beach D. and Lowe S. W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593–602.

Shao J., Sheng H., DuBois R. N. and Beauchamp R. D. (2000) Oncogenic Ras-mediated cell growth arrest and apoptosis are associated with increased ubiquitin-dependent cyclin D1 degradation. *J. Biol. Chem.* **275**, 22916–22924.

Sharma V., Joseph C., Ghosh S., Agarwal A., Mishra M. K. and Sen E. (2007) Kaempferol induces apoptosis in glioblastoma cells through oxidative stress. *Mol. Cancer Therapeut.* **6**, 2544–2553.

Stickney J. T., Bacon W. C., Rojas M., Ratner N. and Ip W. (2004) Activation of the tumor suppressor merlin modulates its interaction with lipid rafts. *Cancer Res.* **64**, 2717–2724.

Strassheim D., Shafer S. H., Phelps S. H. and Williams C. L. (2000) Small cell lung carcinoma exhibits greater phospholipase C-beta1 expression and edelfosine resistance compared with non-small cell lung carcinoma. *Cancer Res.* **60**, 2730–2736.

Tachibana O., Nakazawa H., Lampe J., Watanabe K., Kleihues P. and Ohgaki H. (1995) Expression of Fas/APO-1 during the progression of astrocytomas. *Cancer Res.* **55**, 5528–5530.

Wallach D., Varfolomeev E. E., Malinin N. L., Goltsev Y. V., Kovalenko A. V. and Boldin M. P. (1999) Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu. Rev. Immunol.* **17**, 331–367.

Wang X., Martindale J. L. and Holbrook N. J. (2000) Requirement for ERK activation in cisplatin-induced apoptosis. *J. Biol. Chem.* **275**, 39435–39443.

Weidhaas J. B., Eisenmann D. M., Holub J. M. and Nallur S. V. (2006) A conserved RAS/mitogen-activated protein kinase pathway regulates DNA damage-induced cell death postirradiation in Radelegans. *Cancer Res.* **66**, 10434–10438.

Xia S., Rosen E. M. and Laterra J. (2005) Sensitization of glioma cells to Fas-dependent apoptosis by chemotherapy-induced oxidative stress. *Cancer Res.* **65**, 5248–5255.

