Proapoptotic and proautophagy effect of H1-receptor antagonist desloratadine in human glioblastoma cell lines

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Abstract

Aims: Glioblastomas are aggressive and usually incurable high grade gliomas without adequate treatment. In this study we aimed to investigate the potential of desloratadine to induce apoptosis/autophagy as genetically regulated processes that can seal cancer cell fates.

Materials and methods: All experiments were performed on U251 human glioblastoma cell line and primary human glioblastoma cell culture. Cytotoxic effect of desloratadine was investigated using MTT and CV assays while oxidative stress, apoptosis and autophagy were detected by flow cytometry and immunoblot.

Results: Desloratadine treatment decreased cell viability of U251 human glioblastoma cell line and primary human glioblastoma cell culture (IC50 value 50 μM) trough an increase of intracellular reactive oxygen species and caspase activity. Also, desloratadine decreased the expression of main autophagy repressor mTOR and its upstream activator Akt and increased the expression of AMPK.

Conclusion: Desloratadine exerted dual cytotoxic effect inducing both, apoptosis and mTOR/AMPK dependent cytotoxic autophagy in glioblastoma cells and primary glioblastoma cell culture

Introduction

Gliomas are tumors of central nervous system that arise from glial cells. They make up about 30% of all brain and central nervous system tumors and 80% of all malignant brain tumors. According to the World health organisation (WHO) gliomas can be classified by cell type, grade, origin, and location [1]. Inaccessible localisation as well as chemo and radio resistance of glioma represents a huge problem in therapeutic treatment.

Glioblastoma represents a most aggressive glioma type and has very poor prognosis with survival time period of approximately 1 year [2]. In addition, glioma cells are prone to acquire drug resistance systems. Therefore, there is a need to identify agents which could overcome tumor chemotherapeutic resistance.

Biogenic amine histamine is an organic nitrogenous compound that acts through specific histaminic receptors (H1-H4). Histamine is involved in local immune response as well as in regulation of physiological function in the gastrointestinal tract and neurotransmission. Some previous studies have reported inverse association between tumorigenesis and allergic conditions [3] but also possible protumorigenic histamines' effect. In this line, some data have shown antitumor effects related to H1 receptor antagonists among different tumor types [4–7], that made us to examine potential antitumor effect of desloratadine, Desloratadine is one of H1 receptor antagonists that is frequently used to suppress itch in pruritic diseases, such as urticaria [8]. On the other hand it was shown that it can induce apoptosis in cutaneous T cell lymphoma *in vitro* [9] and express anticancer effects when investigated *in vivo* [10].

When anticancer effect of potentially new chemotherapeutic agent is examined, it is preferred that it does not induce inflammation as a general response to cell death. Therefore, it would be preferable that potentially anticancer agent induces apoptosis and/or autophagy as the cell death mechanisms that are not accompained by inflammation [11].

Apoptosis (programmed cell death type I) is an active process by which cells activate intracellular death program that is mediated by caspase activation and leads to self-destruction[12]. It is widely considered to be one of the most important mechanisms by which anticancer agents accomplish its cytotoxic effect. Therefore, any deficiency in the apoptotic cascade could lead to a drug-resistant phenotype.

Beside apoptosis, as a most investigated mechanism of cell death induced by H1 receptor antagonist [3, 4], some data revealed that H1 antagonists can also induce autophagy [13, 14]. Macroautophagy (referred to here after as autophagy) is a catabolic process used for degradation and recycling of the cell's own unnecessary or dysfunctional components [15]. Autophagy is controlled by AMP activated protein kinase (AMPK), which is a key energy sensor that regulates cellular metabolism in order to maintain energy homeostasis in cells. During nutrient-deprivation, hypoxia, oxidative stress, DNA damage and other stress conditions, autophagy provides energy for maintaining essential cellular metabolism and can interfere with apoptotic/necrotic cell death pathways [16]. Keeping that in mind, it was shown that there is a cross talk between apoptosis and autophagy so that, in certain situations, autophagy can also stimulate apoptosis [17] or can function as an alternative cell-death pathway (programmed cell death type II) [18]. On the other hand, mammalian target of rapamycin (mTOR), cell-growth regulator, is main autophagy repressor which is activated by phosphoinositide 3-kinase (PI3K)/Akt pathway[15]. One of most common used indicator of autophagy vesicles formation is increased expression of LC3II. [19]. There are data showing that H1 antagonists like diphenhydramine and AST–HIS induce autophagy in astrocytes and MCF-7 cells, respectively [13, 14] while deptropin block basal autophagy in human hepatoma cells [20]

Taking into account the great antiapoptotic potential of brain tumor cells [21, 22], and the fact that almost all available anticancer treatments induce cytotoxic effects by means of apoptosis[22] it would be of great importance to investigate also a possible proautophagic effects of potential novel therapeutic treatment.

Since, to the best of our knowledge, there are not any data about potential cytotoxic effect of H1 antagonists on glioblastoma cells, we aimed to investigate the potential cytotoxic effect of tricyclic antihistaminic, desloratadine, on glioblastoma cells in culture, as well as the mechanism underlying its potential cytotoxic effect.

Material And Methods

Cell culture and chemicals

All chemicals were from Sigma Aldrich unless otherwise stated.

U251 (human glioblastoma cell line), was obtained from European Collection of Cell Cultures ECACC. This cell line is not listed as a commonly misidentified cell line by International Cell Line Authentication Committee. Cells were maintained at 37°C degrees, in a humidified atmosphere with 5% CO2. Cells were cultured in 20mM Hepes buffered RPMI-1640 cell culture medium supplemented with 5% fetal calf serum, and 2 mM glutamine. All cells were passage 10–15 and for experiments were prepared using the conventional trypsinization procedure with trypsin /EDTA. After the trypsinization cells were seeded in 96 well flat bottom plates ($15x10^4$ cells/well) for viability assays and in 6 well flat bottom plates ($3x10^5$ cells/well) for flow cytometry analysis. For immunoblot analysis cells were seeded in Petri dishes (10 cm, $2.5x10^6$ cells). After seeding, cells were rested for 24h and than treated with desloratadine which was dissolved in cell culture medium (concentrations were from 7.8–1000 μM). All experiments were performed after 24h cells' treatment except Western Blot analysis which was performed after 4h, 6h and 18h treatment with desloratadine.

Primary glioblastoma cell line

The primary glioblastoma cell culture was established from the tumor tissue collected from patients with WHO grade IV glioblastoma (Clinic of Neurosurgery, Department of Neurooncology, Clinical Center of Serbia, Belgrade). Prior informed consent was obtained in written form from all patients, and all procedures were approved by the institutions' Ethical Committee in accordance with the Declaration of Helsinki. Isolation of human primary glioblastoma cells was performed according to the previously described procedure [23].

Cell culture medium (20mM Hepes buffered RPMI-1640 supplemented with 10% fetal calf serum, and 2mM glutamine) was changed after 24h and then every 48h until the cells reached 80%. Cells were detached by conventional trypsinization procedure and passaged 2–3 times in this manner, before the experimental procedure. Cells were rested for 24h in cell culture medium and then treated with desloratadine and/or autophagy inhibitors bafilomycin A1, hydroxychloroquine and NH4Cl, as described in Results and Figure legends.

Determination of cell viability

Cell viability was assessed using Crystal violet assay, to stain viable, adherent cells and MTT assay to measure the activity of mitochondrial dehydrogenases [24]. The absorbances were measured in an automated micro plate reader at 570nm (Sunrise, TECAN, UK) and the results are presented as relative to the control value (untreated cells).

Reactive oxygen species (ROS) measurement

Intracellular production of ROS was determinate by measuring the intensity of green fluorescence, emitted by redox-sensitive dye dihydrorodamine 123 (DHR; Invitrogen), using flow cytometry analysis (FACS Calibur flow cytometer, BD, Heidelberg, Germany). The results are presented as fold change in fluorescence intensity compared to that of untreated (control) cells, which was arbitrarily set to 1.

Caspase activity

Caspase activation was measured using flow cytomerty after labelling the cells with a cell permeable FITC-conjugated PAN caspase inhibitor (ApoStat R&D systems) according to manufacturer's instructions. The increase in green fluorescence (FL1) is presented as a fold change in fluorescence intensity compared to that of untreated (control) cells, which was arbitrarily set to 1.

Measurement of intracellular acidification

The acidic vesicles (i.e. lysosomes, autophagolysosomes) were visualized by supravital stain acridine orange (AO) under the inverted fluorescent microscope (Leica Microsystems DMIL, Wetzlar, Germany) using Leica Microsystems DFC320 camera and Leica Application Suite software (version 2.8.1). Alternatively, acridine orange-stained cells were also analyzed on a FACSCalibur flow cytometer using Cell Quest Pro software. Accumulation of acidic vesicles was quantified as red/green fluorescence ratio (mean FL3/FL1).

Apoptosis determination

Apoptotic cell death was analyzed by double staining with annexin (V–FITC) and propidium iodide (PI) that emitted green (FL1) and red (FL2) fluorescence, respectively. Staining was performed according to the manufacturer's instructions (Abcam, UK). A fluorescence of annexin/PI- and PI-stained cells was analyzed using a FACS Calibur flow cytometer (BD, Heidelberg, Germany). The percentages of viable (annexin-/PI-), early apoptotic (annexin+/PI-), and late apoptotic/necrotic (annexin+/PI+) cells, were determined using Cell Quest Pro software (BD, Heidelberg, Germany).

Immunoblot analysis

After incubation and treatment cells were lysed and prepared for immunoblot analysis [23]. Membranes were incubated with primary antibody for LC3II, phospho-AMPK (Thr127), AMPK, phospho-AKT (Ser 473), AKT, phospho-mTOR (Ser 2448), mTOR, actin and peroxidase-conjugated goat anti-rabbit IgG (all from Cell Signal Technology), as the secondary antibody. Specific protein bands were visualized using Amersham ELC reagent (GE Healthcare). The protein levels were quantified using densitometry (Image Lab program) and expressed relative to actin (LC3II) or corresponding total protein signal (phospho-AMPK, phospho-AKT, phospho-mTOR). The results are presented as fold change in signal intensity compared to that of untreated (control) cells at the same time point, which was arbitrarily set to 1.

Statistic analysis

The statistical significance of the differences was analyzed by Student's t-test or one way analysis of variance (ANOVA) followed by Student-Newman Keuls test. A p value of less than 0.05 was considered significant.

Results

Desloratadine exerts antitumor activity against U251 cells

When used in different concentrations (7.8–1000 μM), desloratadine, decreased U251 cell viability in dose dependent manner, with IC50 value of 50 μM after 24h incubation. There was no statistical significant difference between results obtained with both viability tests (CV and MTT) (Fig. 1A).

Cytotoxic effect of desloratadine against U251 cells was further verified using phase contrast microscopy (Fig. 1B). Morphological analysis of cells treated with desloratadine showed decrease in the number of live cells (polygonal, dark, adherent cells) and an increase in the number of dead cells (round, white non-adherent cells). Untreated, control cells had typical cobble stone like appearance.

Desloratadine induce oxidative stress and caspase dependent apoptosis of U251 cells

Since desloratatine significantly increased production of ROS in dose dependent manner (1.26 and 1.4 fold increase against control cells when used in concentrations of 25 μM and 50 μM ,respectively), that was verified with DHR staining, we assumed that oxidative stress is a contributing factor of its observed cytotoxic effect (Fig. 2A)

Measurement of caspase activation, using fluorescent labelled pan-caspase inhibitor ApoStat after 24h treatment, showed significant caspase activation. When cells were treated with 25 μM and 50 μM of desloratadine, the caspase activity increased 1.4 and 1.3 times relatively to control cells, respectively (Fig. 2B). These results could indicate that desloratadine induces apoptosis which is caspase dependent.

Further, apoptotic cell death was investigated and verified using Ann/PI staining. We showed that desloratadine induced an increase in the percentage of Ann positive cells in dose dependent manner. Namely, number of Ann positive cells treated with 12.5 μM, 25 μM and 50 μM of desloratadine was 8.65%, 16.79% and 47.08% respectively (Fig. 2C). These data indicate that desloratadine induces apoptotic cell death in U251 cells.

Desloratadine induce AMPK/mTOR dependent autophagy of U251 cells

The presence of autophagy was investigated first qualitatively by fluorescent microscopy. U251 cells treated with desloratadine (50 μM) had increased number of intracytoplasmic acidic vesicles (lysosomes/ autophagolysosomes) that correspond to orange-red fluorescent spots in green cytoplasm, compared to control (untreated cells) (Fig. 3A).

To quantify acidic vesicles content, we measured the increase of red/green (FL3/FL1) fluorescence ratio, using flow cytometry. After 24h treatment with desloratadine (50 μM) flow cytometry analysis revealed significant increase in FL3/FL1 fluorescence ratio (FL3/FL1 = 1.3) comparing to control cells (FL3/FL1 = 1) (Fig. 3B). Given data suggest the increase in acidic intracytoplasmic vesicles content.

As AO staining is not specific for autophagolysosomes only, since it can label also other acidic vesicles, we further performed immunoblot analysis to confirm the presence of autophagy related proteins and to

define intracellular signalling pathways involved in autophagy induction. Therefore, we measured the expression of LC3II protein, which is a constituent of autophagosome membrane. It is formed through the conversion of soluble cytoplasmic form of LC3I to membrane form, LC3II [19], during the maturation of autophagosomes [25]. Also, proteins of mTOR signalling pathway were measured. After 4h, 6h and 18h treatment with desloratadine (50 μM) expression of pAKT, pmTOR, and pAMPK were analyzed, while expression of LC3II was analyzed following 18h and 24h treatment with desloratadine (50 μM). Immunoblot analysis revealed that, under the treatment with desloratadine (50 μM), the expression of main autophagy repressor mTOR and its upstream activator Akt were decreased, while main mTOR inhibitor, AMPK, was increased after 6h and 18h incubation (Fig. 3C). Increase in one of autophagy markers, LC3II was observed after 18h and 24h incubation (Fig. 3D). These results indicate that desloratadine induces AMPK/mTOR dependent autophagy .

To define whether the autophagy is the type of cell death or cell protection mechanism, U251 cells were simultaneously treated with desloratadine (50 μM) and one of well known autophagy inhibitors: bafalomycin (5 nM), chloroquine (10 mM) or ammonium chloride (5 mM). After 24h incubation, the presence of inhibitors reduced cytotoxity of desloratadine suggesting that autophagy in U251 cells contributed to its cytotoxic effects (Fig. 3E).

Desloratadine exerts cytotoxic effect and induces oxidative stress and autophagy in primary glioblastoma cells

Additionally, antitumor effect of desloratadine was also confirmed on primary human glioblastoma cell culture. Viability assays revealed decrease in cell viability in dose dependent manner, with IC50 value of 50 μM (Fig. 4A) while phase-contrast microscopy revealed same morphological changes as in U251 cell line that indicated an increase in the number of dead cells (round, white non-adherent cells) (Fig. 4B).

Acidic vesicles content was quantified after AO staining as the increase of FL3 red/FL1 green fluorescence ratio, using flow cytometry analysis. After 24h treatment with desloratadine (50 μM) flow cytometry analysis revealed significant increase in FL3/FL1 fluorescence ratio (FL3/FL1 = 3) comparing to control cells (FL3/FL1 = 1) (Fig. 4C). The presence of acidic vesicles was further confirmed by fluorescent microscopy of AO stained glioblastoma cells which revealed an increase number of intracytoplasmic acidic vesicles (lysosomes/ autophagolysosomes) after 24h treatment with desloratadine (50 μM) (Fig. 4D).

Additionally, the presence of autophagy was confirmed by measuring the expression of LC3II protein. After 4h, 6h and 18h treatment with desloratadine (50 μM) increase in LC3II level was observed (Fig. 4E).

DHR staining demonstrated that after 24h treatment with desloratadine, ROS production increased in dose dependent manner. Namely, production of ROS increased 2 and 3.9 times compared to control in the presence of 25 μM and 50 μM of desloratadine, respectively (Fig. 4F).

Unfortunately, using Ann/PI and pancaspase inhibitor, we did not detect either apoptosis either caspase activity under the treatment of human glioblastoma primary cell culture with desloratadine (data not shown).

Discussion

In the present study we demonstrate that H1 antagonist desloratadine decreases viability of glioblastoma cells in culture, through various mechanisms, including increase in reactive oxygen species production, induction of apoptosis and autophagy. Aside from a report that other H1 antagonists can induce apoptosis and autophagy [3, 6, 13, 14], this is the first study clearly demonstrating desloratadine induced activation of Akt/mTOR-dependent cytotoxic autophagy in tumor cells.

Keeping in mind that glioblastoma is an aggressive tumor that has the highest rate of mortality among all malignant brain tumors [26] there is an huge requirement to identify novel target molecules to which more effective therapeutic approaches can be developed. It was already shown that H1 antagonists can exert cytotoxic effects through the induction of both, apoptosis and autophagy [3, 6, 13, 14, 27]. Even the histamine deficiency can induce apoptosis [28] suggesting the role of histamine receptor activity in cell survival. In the current study we investigated the potential cytotoxic effects of H1 receptor antagonist, desloratadine, on glioblastoma cell line and primary human glioblastoma cell culture.

Glioblastoma cells express great chemoresistance [29] that can be supported by a presence of membranous ABC (ATP-binding Cassette) superfamily of efflux pumps located on blood-brain barrier [30]. Also, genetic instability caused with numerous mutations, deletions and genetic amplifications contributes its' chemoresistance[31]. Having in mind all this complex glioblastoma characteristics that lead to their great biological diversity, there is a need for combined therapeutic approaches, targeting more than one signalling pathway. The results of our study contribute to this approach, since desloratadine affected both, apoptosis and autophagy, as two different programmed cell death mechanisms. In addition to already known desloratadine cytotoxic effect against cutaneous T-cell lymphoma cell lines, EJ and SW780 cells [9, 27] our results clearly shown dose dependent cytotoxic activity of desloratadine against U251 cell line. Nevertheless, the obtained IC50 value for desloratadine is in accordance with its cytotoxic activity on non small cell lung cancer (NSCLC) cell lines [10]. Its cytotoxic effects are, even partially, achieved through the induction of ROS overproduction which is a well-known trigger that could induce cell death [32]. Moreover, we found that desloratadine induced the externalization of phosphatidylserine in cell membrane, together with an increase in caspase activation, both hallmarks of apoptosis. Given data are in accordance with studies taken by Plekhova et al., and Ma et al., where Ann + macrophages as well as bladder cancer EJ and SW780 cells were detected after desloratadine treatment, respectively [27, 33]. Also, it was shown that desloratadine can induce apoptosis in cutaneous T-cell lymphoma cell lines through inhibition of STAT3 and c-Myc activities. Additionally, caspase dependent proapoptotic activity was previously reported for other H1 antagonists such as diphenhydramine and meclizine [5, 7].

It is known that autophagy in tumour cells can either promote apoptosis or serve as mechanism of programmed cell death type II. Several reports provided evidence that spontaneous autophagy is reduced in glioblastoma [32, 34] suggesting that this could be responsible for its considerable malignant potential. In contrast, there are data that show autophagy as a prosurvival mechanism that protects cancer cells from apoptotic or necrotic cell death induced by different anticancer drugs [17]. In the light of all the foregoing considerations we were interested to examine this process using glioblastoma cell line.

We have shown that desloratadine induces autophagy in U251 glioblastoma cells through increased expression of acidic cytosolic vesicles, both qualitatively and quantitatively. Given data are additionally confirmed with increased expression of autophagosome-associated LC3II protein. Ma et al., also impaired expression of autophagy-related proteins, such as Beclin 1, p62 and LC3I/II in EJ and SW780 cells after treatment with desloratadine [27]. After diphenhydramine treatment acidic vacuoles were detected in astrocytes, whitch is confirmed with increased LC3-I to LC3-II conversion[13] Autophagy followed with increased expression of LC3II protein was also reported for some other H1 antagonists [6, 14]. Furthermore, we looked towards the intracellular signalling pathway underlying autophagic process of U251 glioblastoma cells induced by desloratadine. AMPK is one of the positive regulators of autophagy that stimulates autophagy in response to energy depletion (increased AMP/ATP ration) [35] and that way connecting cellular energy homeostasis and autophagy. Beside AMPK, we looked toward activity of AMPK downstream target, mTOR, as well as prosurvival signalling molecule Akt involved in stimulation of protein synthesis and cell proliferation. Akt signalling pathway is usually regarded as independent signalling route from AMPK signalling pathway, and as alternative pathway that regulates autophagy [36]. In our experimental model we observed the up regulation of AMPK and down regulation of mTOR and Akt indicating that desloratadine induced autophagy of U251 cells is mTOR/AMPK dependent. Astemizol is the other histamine antagonist that induced autophagy in breast cancer cells [14]. Beside autophagy and similarly to desloratadine, astemizol also induced apoptosis through caspase activation and increased production of ROS. On the other hand, there are data indicating that antihistamine deptropin blocked basal autophagy which is experimentally confirmed through increased LC3II expression while Akt,AMPK, vacuolar protein sorting 34 (VPS34) and Atg7 expression was not changed after treatment in human hepatoma cells[20] Having in mind that autophagy could be either prosurvival or cell death mechanism, using simultaneous treatment with different autophagy inhibitors and desloratadine, we have shown that the autophagy in glioblastoma cells induced by desloratadine is cytotoxic. Together with previously reported finding that glioblastomas cells have reduced spontaneous autophagy, this induction of cytotoxic autophagy with desloratadine could be of great importance in modulation of current chemo-radiation treatment. The effect of autophagy modulation on glioblastoma radio sensitivity was intensively investigated previously [37] and, even still not completely clear, it is evident that autophagy modulation offers a promising, novel approach to glioblastoma treatment.

In addition to desloratadine effects observed in U251 glioblastoma cell line, we showed also similar patterns of desloratadine action against human glioblastoma primary cell culture. Its cytotoxic effect was performed through induced ROS production that, possibly initiated autophagy verified through an increase in number of acidic vesicles as well as an increase in LC3II autophagosome-associated protein.

The observed results clearly demonstrate that desloratadine induces apoptosis accompanying with cytotoxic mTOR/AMPK dependent autophagy. This dual antitumor potential of desloratadine is particularly important for tumor cells that express great antiapoptotic potential, such as cells of glioblastoma.

Declarations

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Disclosure of interest

The authors declare that they have no conflict of interest.

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Figures

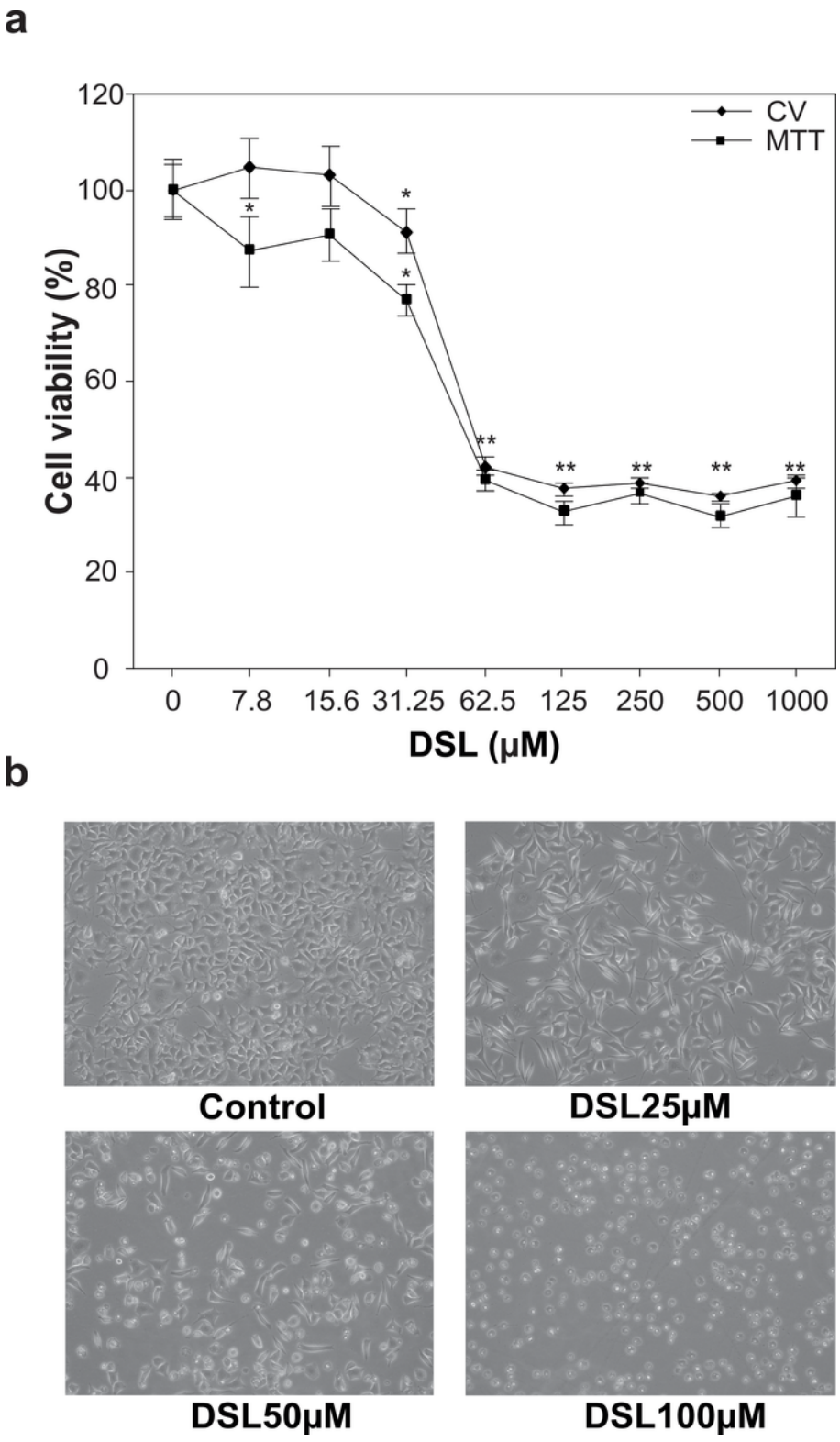

Figure 1

Desloratadine reduces viability of U251 glioma cells in dose dependent manner.

U251 cells were incubated for 24h with different concentrations of desloratadine (7.8-1000 μM) and the cell viability was estimated by MTT and CV assays (a). Cell morphology was examined by phase contrast microscopy after 24h treatment with different concentrations of desloratadine (b). Data are presented as

mean ± SD values of three independent experiments (*p < 0.05 compared to control cells; **p<0.01 compared to control cells).

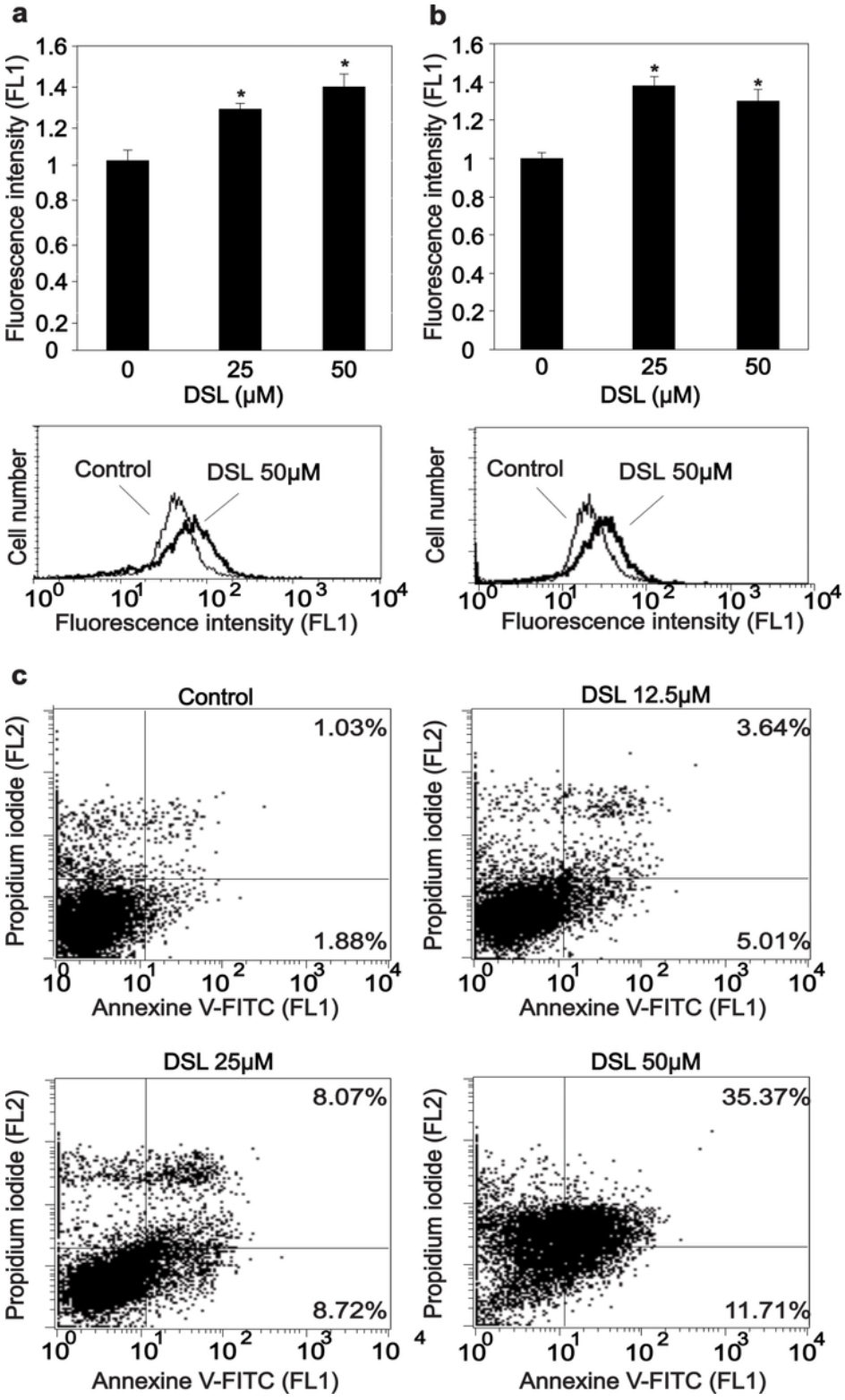

Figure 2

Desloratadine induce oxidative stress and caspase dependent apoptosis in U251 cells. Cells were treated with desloratadine for 24h and ROS production (a) caspase activation (b) and apoptotic/necrotic cell

death (c) were examined by flow cytometry using DHR, Apostat and annexin/PI staining respectively. Data are presented as mean ± SD values of three independent experiments (*p < 0.05 compared to control cells).

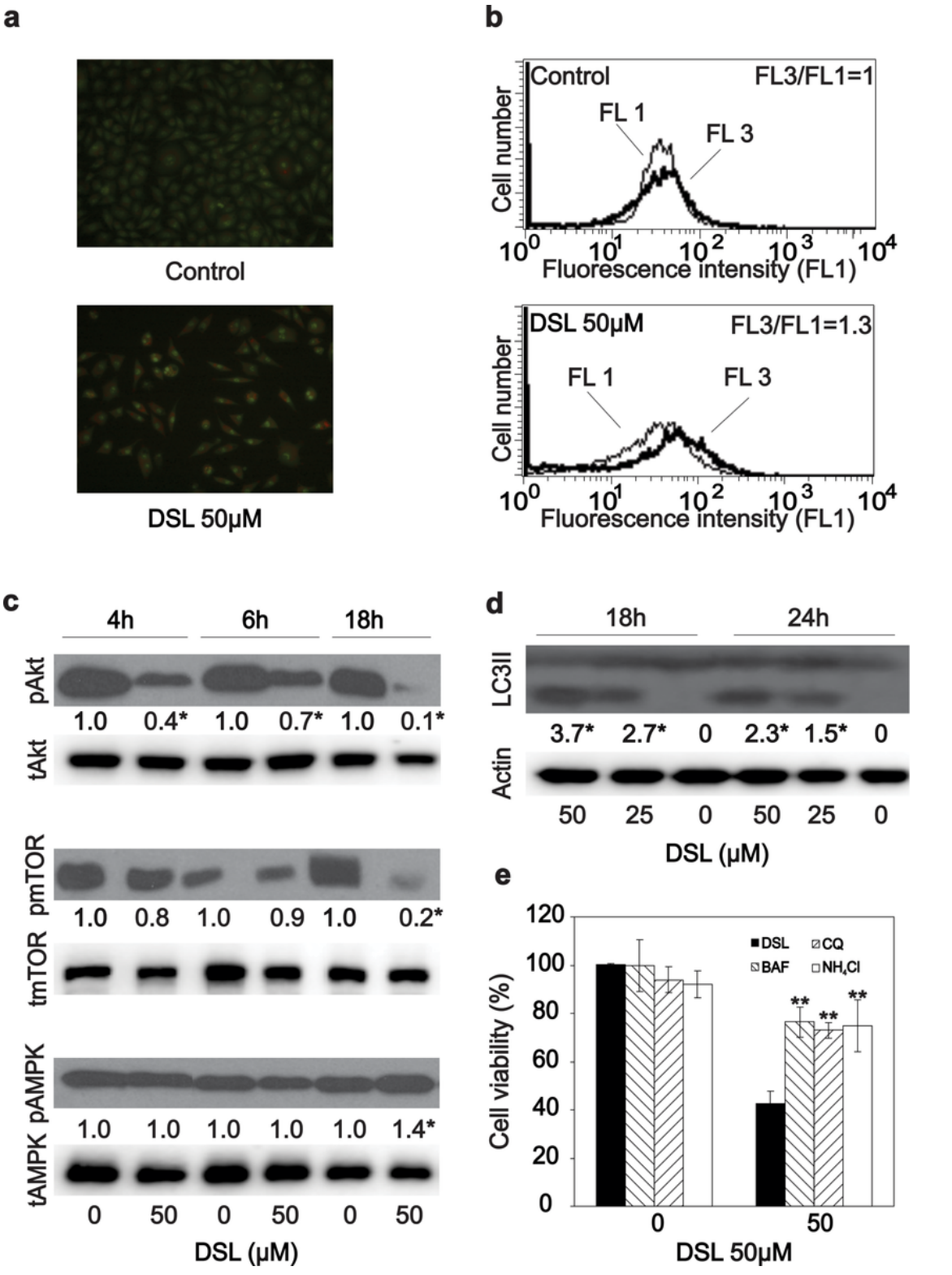

Figure 3

Desloratadine induce AMPK/mTOR dependent autophagy in U251 cells.

The presence of intracytoplasmic acidic vesicles after 24h treatment with desloratadine (50 μM) was verified by fluorescent microscopy after AO staining (a) while quantification of acidic vesicles content through FL3/FL1 ratio was analyzed by flow cytometry (b). The levels of phosphorylated/total Akt, mTOR, AMPK relative to total corresponding protein expression (c) and LC3II relative to actin (d) were determined by immunoblotting for the indicated time periods and signal intensity are presented below the protein bands. Representative blots from three independent experiments are presented. To analyze the nature of autophagy induced with desloratadine, cell viability of U251 cells simultaneously treated with desloratadine (50 μM) and one of autophagy inhibitors: bafalomycin (5 nM), chloroquine (10 μM) and ammonium chloride (5 mM) was measured using MTT assay (e). Data are presented as mean ± SD values of three independent experiments (*$p < 0.05$ compared to control cells).

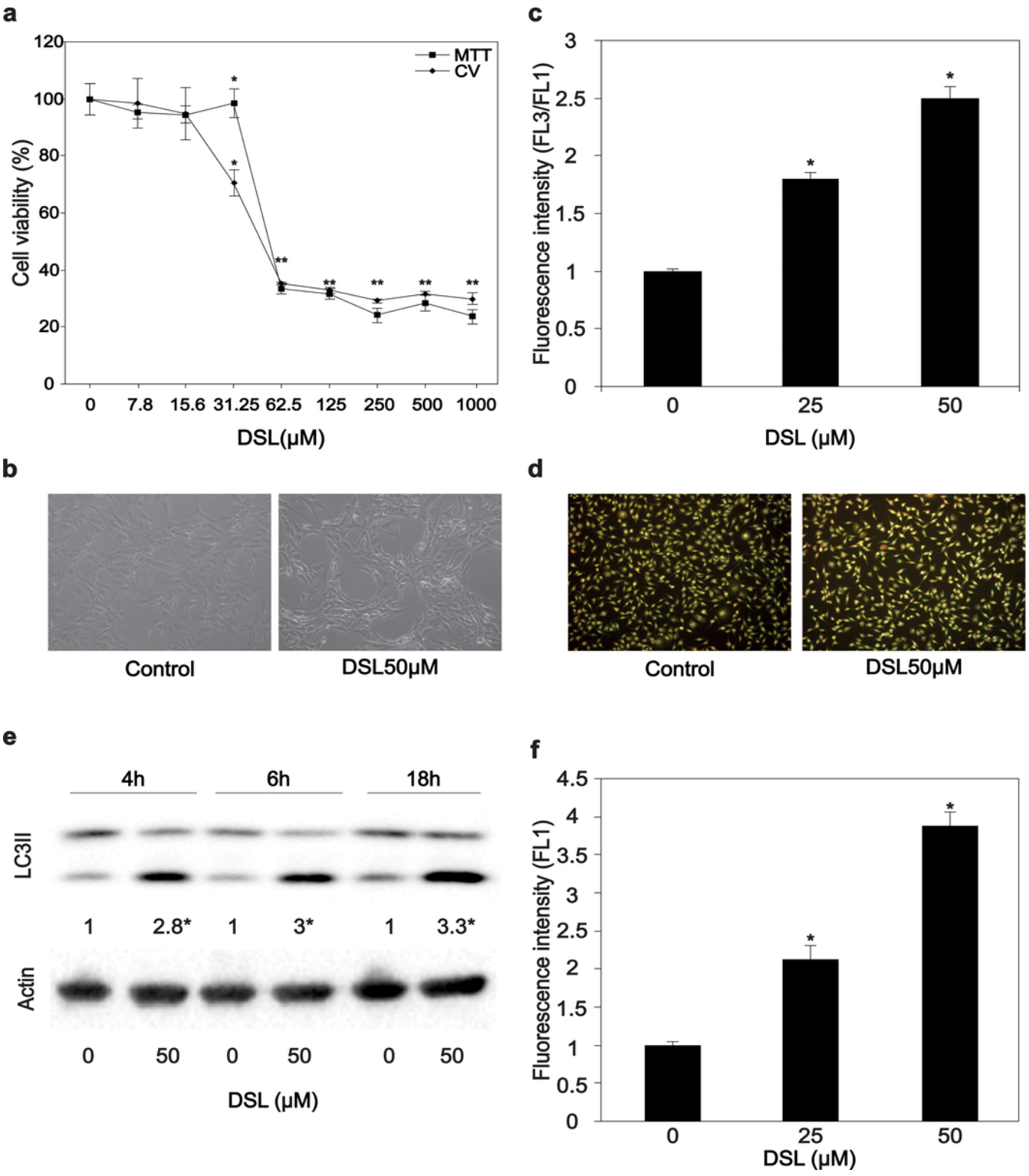

Figure 4

Desloratadine exerts cytotoxic effect and induces oxidative stress and autophagy in primary human glioblastoma cells.

Primary human glioblastoma cells were incubated for 24h with different concentrations of desloratadine (7.8-1000 μM) and the cell viability was estimated by MTT and CV assays (a). Cell morphology was

examined by phase contrast microscopy after 24h treatment with desloratadine (50 μM) (b). Acidic vesicle content was verified by FL3/FL1 ratio using flow cytometry (c) while the presence intracytoplasmic acidic vesicles was verified by fluorescent microscopy after AO staining (d). The levels of autophagy related protein LC3II relative to actin (e) in cells treated with desloratadine (50 μM) was determined by immunoblotting for the indicated time periods and signal intensity are presented below the protein bands. Representative blots from three independent experiments are presented. ROS production (f) was measured by DHR staining using flow cytometry. Data (A, C, F) are presented as mean ± SD values of at least three independent experiments (*$p < 0.05$ compared to control cells; **$p<0.01$ compared to control cells).