www.elsevier.com/locate/yexcr

The combination of glutamate receptor antagonist MK-801 with tamoxifen and its active metabolites potentiates their antiproliferative activity in mouse melanoma K1735-M2 cells

Mariana P.C. Ribeiro, Isabel Nunes-Correia, Armanda E. Santos, José B.A. Custódio

PII: S0014-4827(13)00477-1
DOI: http://dx.doi.org/10.1016/j.yexcr.2013.11.002
Reference: YEXCR9466

To appear in: *Experimental Cell Research*

Received date: 19 September 2013
Revised date: 28 October 2013
Accepted date: 8 November 2013

Cite this article as: Mariana P.C. Ribeiro, Isabel Nunes-Correia, Armanda E. Santos, José B.A. Custódio, The combination of glutamate receptor antagonist MK-801 with tamoxifen and its active metabolites potentiates their antiproliferative activity in mouse melanoma K1735-M2 cells, *Experimental Cell Research,* http://dx.doi.org/10.1016/j.yexcr.2013.11.002

The combination of glutamate receptor antagonist MK-801 with tamoxifen and its active metabolites potentiates their antiproliferative activity in mouse melanoma K1735-M2 cells

Mariana P. C. Ribeiro[a,b], Isabel Nunes-Correia[c], Armanda E. Santos[a,b]*, and José B.A. Custódio[a,b]

[a]Center for Neuroscience and Cell Biology, University of Coimbra, 3000-354 Coimbra, Portugal;

[b]Laboratory of Biochemistry, Faculty of Pharmacy, University of Coimbra, 3000-548 Coimbra, Portugal;

[c]Center for Neuroscience and Cell Biology, Flow Cytometry Unit, University of Coimbra, 3000-354 Coimbra, Portugal;

Corresponding author: Armanda E. Santos, Laboratório de Bioquímica, Faculdade de Farmácia, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

e-mail: aesantos@ci.uc.pt

Telephone and fax number: +351 239 488 400, +351 239 488 503

Abbreviations

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA receptor; APV, D-(-)-2-amino-5-phosphonopentanoic acid; BrdU, 5-bromo-2'-deoxyuridine; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EDX, endoxifen; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GluR, glutamate receptor; iGluR, ionotropic glutamate receptor; KA, kainate; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; mGluR, metabotropic glutamate receptor; NAD, nicotinamide adenine dinucleotide; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline; NMDA, *N*-methyl-D-aspartate; NMDAR,

NMDA receptor; OHTAM, 4-hydroxytamoxifen; PBS, phosphate-buffered saline; SRB, sulforhodamine B; TAM, tamoxifen; TMB, 3,3',5,5'-tetramethylbenzidine.

Abstract

Recent reports suggest that *N*-methyl-D-aspartate receptor (NMDAR) blockade by MK-801 decreases tumor growth. Thus, we investigated whether other ionotropic glutamate receptor (iGluR) antagonists were also able to modulate the proliferation of melanoma cells. On the other hand, the antiestrogen tamoxifen (TAM) decreases the proliferation of melanoma cells, and is included in combined therapies for melanoma. As the efficacy of TAM is limited by its metabolism, we investigated the effects of the NMDAR antagonist MK-801 in combination with TAM and its active metabolites, 4-hydroxytamoxifen (OHTAM) and endoxifen (EDX). The NMDAR blockers MK-801 and memantine decreased mouse melanoma K1735-M2 cell proliferation. In contrast, the NMDAR competitive antagonist APV and the AMPA and kainate receptor antagonist NBQX did not affect cell proliferation, suggesting that among the iGluR antagonists only the NMDAR channel blockers inhibit melanoma cell proliferation. The combination of antiestrogens with MK-801 potentiated their individual effects on cell biomass due to diminished cell proliferation, since it decreased the cell number and DNA synthesis without increasing cell death. Importantly, TAM metabolites combined with MK-801 promoted cell cycle arrest in G1. Therefore, the data obtained suggest that the activity of MK-801 and antiestrogens in K1735-M2 cells is greatly enhanced when used in combination.

Keywords: glutamate receptor antagonists; antiestrogens; melanoma; cell proliferation.

Introduction

Emerging evidence indicates that melanoma is a very heterogeneous malignancy, with several variants and with multiple signaling pathways contributing to cell proliferation constitutively activated (Herlyn, 2009). Therefore, in order to target such diversity, we need to develop combinations of drugs with specific and complementary mechanisms of action (Herlyn, 2009; Ko and Fisher, 2011).

Glutamate, the major excitatory neurotransmitter of the mammalian central nervous system, activates two classes of glutamate receptors (GluRs), the ionotropic (iGluRs) and metabotropic (mGluRs) receptors. The iGluRs form ion channels, while the mGluRs belong to the superfamily of G protein-coupled receptors (Teh and Chen, 2012). The iGluRs are divided into three groups based on structural and pharmacological similarities, and are named *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and kainate (KA) receptors, according to the type of synthetic agonist that activates them. So far, eight members of mGluRs have been identified, which are grouped in three classes based on sequence homology and downstream signaling pathways.

The fact that both neuronal embryonic progenitor cells and tumor cells have propensity to proliferate and migrate led to the investigation of the role of glutamate and its receptors on the proliferation and migration of cancer cells. It has been reported that GluR subunits are differentially expressed in a variety of tumor cell lines (North et al., 1997; Stepulak et al., 2009; Brocke et al., 2010; North et al., 2010a, b; Stepulak et al., 2011) and in samples of human tumor tissues (North et al., 2010a, b). The knockdown of selected GluR subunits has also been shown to modulate cancer cell proliferation and invasive behavior (de Groot et al., 2008; Luksch et al., 2011). Moreover, NMDA receptor (NMDAR) and AMPA receptor (AMPAR) antagonists inhibit the proliferation and migration of tumor cells and enhance the effects of cytostatic drugs, such as cyclophosphamide and thiotepa, *in vitro* and *in vivo* (Stepulak et al., 2005, 2007; North et al., 2010a, b; Rzeski et al., 2011; Stepulak et al., 2011).

Beyond the role played by mGluR signaling in melanoma cells (Marín et al., 2006; Namkoong et al., 2007; Abdel-Daim et al., 2010; Lee et al., 2011), recent reports also suggest a role for iGluRs, since functional NMDARs are expressed in this type of cells and the NMDAR antagonist MK-801 was shown to inhibit the migration and proliferation of melanoma cells and to decrease their growth *in vivo* (Song et al., 2012). In addition, the AMPAR antagonist CFM-2, as well as the NMDAR antagonists memantine and MK-801 have been shown to alter melanocyte morphology, indicating that glutamate signaling may be relevant in melanocyte regulation (Hoogduijn et al., 2006).

On the other hand, it has been reported that tamoxifen (TAM), a selective estrogen receptor (ER) modulator widely used in the treatment and prevention of breast

cancer, also decreases the growth and migration of melanoma cells (Kanter-Lewensohn et al., 2000; Matsuoka et al., 2009; Ribeiro et al., 2013) and sensitizes melanoma cells to other chemotherapeutic agents (Flaherty et al., 1996; McClay et al., 1997). The biological activity of TAM is mediated by two active metabolites, 4-hydroxytamoxifen (OHTAM) and endoxifen (EDX), generated via cytochrome P450 (CYP) enzymes, namely CYP3A4 and CYP2D6 (Kiyotani et al., 2012). Recent studies point to an association between CYP2D6 polymorphisms and the clinical outcome in women treated with TAM (Schroth et al., 2009; Lammers et al., 2010). Furthermore, it was shown that the coadministration of CYP2D6-inhibiting medication can limit the efficacy of TAM therapy (Kelly et al., 2010). Therefore, the use of TAM active metabolites may present strong advantages over the utilization of the prodrug, as it would avoid the variability related with TAM metabolism, leading to a more reliable therapeutic outcome.

Based on these findings, we investigated the effects of iGluR antagonists on the proliferation of a highly invasive mouse melanoma cell line (K1735-M2). Additionally, since the combination of drugs with complementary mechanisms of action can provide superior therapeutic efficacy using lower concentrations, with the advantage of reducing the side effects of chemotherapy, we evaluated the effects of MK-801 in combination with antiestrogens on cell proliferation as well. We show that the NMDAR channel pore blockers, MK-801 and memantine, decrease mouse K1735-M2 melanoma cell proliferation due to decreased cell division. Moreover, at the concentrations used, the combined treatment of MK-801 with antiestrogens, and particularly with TAM metabolites, strongly enhances the antiproliferative effects induced by the compounds individually, supporting the view that these drugs in association may be useful in malignant melanoma therapy.

Materials and Methods

Reagents

MK-801, memantine, TAM, OHTAM and EDX were obtained from SIGMA-Aldrich (St Louis, MO, USA). 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline(NBQX) was purchased from Tocris. D-2-amino-5-phosphonovaleric acid (APV), Dulbecco's modified Eagle's medium (DMEM) and

antibiotic/antimycotic solution (10 000 units penicillin, 10 mg streptomycin, 25 µg amphotericin B per mL) were purchased from SIGMA-Aldrich (St Louis, MO, USA), Fetal Bovine Serum (FBS) and trypsin were obtained from Gibco, Invitrogen Life Technologies (Carlsbad, California, USA). All of the other chemicals were purchased from SIGMA-Aldrich (St Louis, MO, USA) and were of the highest grade of purity commercially available. GluR antagonists were kept in aqueous stocks. TAM and OHTAM stock solutions were prepared in absolute ethanol while EDX was prepared in dimethyl sulfoxide (DMSO).

Cell culture

K1735-M2 mouse melanoma cells (kindly offered by Dr. Paulo Oliveira, Center for Neurosciences and Cell Biology, Department of Zoology, University of Coimbra, Portugal) were cultured in DMEM, supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution, and kept in a humidified atmosphere with 5% CO_2/95% air, at 37 ºC.

Cells were plated with a density of 6.1×10^4 cells/cm^2 and24 h after plating, the GluR antagonists and/or the antiestrogens were added to the cultures from diluted stocks, except in the control condition where the vehicle was added.

Sulforhodamine B (SRB) assay

The effects induced by the drugs on melanoma cell cultures were determined using the SRB assay, which is based on the binding of SRB to the basic amino acids of cellular proteins (Holy et al., 2006). At selected time points, the cell culture was fixed with absolute methanol containing 1% acetic acid, and stored at −20 ºC overnight. The methanol was then decanted and the plate air-dried. The SRB solution (0.5% in 1% acetic acid) was added to each well, and the plate incubated at 37 °C for 1 h. The cells were rinsed with 1% acetic acid, air-dried, and the bound dye eluted with 10 mM Tris buffer, pH 10. The absorbance was measured in a Synergy HT plate reader at 540 nm, providing an estimate of the total protein mass (biomass) which is related to the cell number. The absorbance obtained in control cultures was considered 100%. Experiments were performed in triplicates for each independent experiment.

Cell viability assessment by trypan blue dye exclusion

Cell viability was investigated by staining cells with trypan blue (Houben et al., 2009). At designated time points, adherent cells were trypsinized, centrifuged at 1 000 rpm for 5 min and treated with 0.4% trypan blue for 2-3 min and then counted in a hemocytometer under a transmitted light microscope. Cells presenting a blue stained cytoplasm were considered as dead cells; cells excluding the dye were considered as viable. The number of independent experiments is indicated in figure legends.

Lactate dehydrogenase (LDH) assay

LDH is a cytosolic enzyme that is released into the extracellular medium following the loss of cell membrane integrity (Vieira et al., 2010). Thus, we investigated the ability of the compounds used in this study to induce melanoma cell death by determining the LDH activity in the cell medium.The culture medium was collected 72 h after incubation with the drugs and centrifuged at 14 000 rpm for 10 min at 4 ºC. An aliquot of supernatant (100 µL) was incubated with a substrate mixture containing 40 µM lactate in perchloric acid 3%, and 3.6 mM nicotinamide adenine dinucleotide (NAD^+) in tris-hydrazine buffer [80 mM tris, 400 mM hydrazine, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 9.5]. LDH activity was determined by an enzymatic reaction whereby the NAD^+ is reduced to NADH by oxidation of lactate to pyruvate. Thus, the amount of NADH is directly related to LDH activity in the supernatant. Absorption of NADH was measured at 340 nm. The LDH activity is expressed as the ratio between the LDH activity in the extracellular medium and the total LDH activity obtained from the supernatant of cells lysed with Triton X-100, which was considered as 100%. Experiments were performed in duplicates for each independent experiment.

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay

Melanoma cell proliferation was monitored through the evaluation of BrdU incorporation during DNA synthesis in proliferating cells. For this purpose, the Cell Proliferation ELISA, BrdU, colorimetric kit (Roche) was used according to the manufacturer's protocol. After 48 h of incubation with the drugs, cultured cells were placed in BrdU-labeling solution for 90 min. Afterwards the cells were fixed and the DNA denaturated with the FixDenat solution, provided with the kit, and then incubated with a monoclonal antibody conjugated with peroxidase (anti-BrdU-POD) to bind BrdU

in the newly synthesized DNA. The immune complexes were detected using the 3,3',5,5'-tetramethylbenzidine (TMB) substrate and the absorbance was measured in a Synergy HT plate reader at 370 nm. The absorbance values correlate to the amount of DNA synthesis and, therefore, to the number of proliferating cells. The experiments were carried out in triplicate for each independent experiment and the absorbance obtained in control cultures was considered as 100%.

Cell cycle analysis by flow cytometry

The effects of the drugs on cell cycle were monitored by flow cytometry (Carmo et al., 2011). Cells were plated in 6-multiwell plates and incubated with MK-801, TAM, OHTAM and EDX for 48 h. At the end of the incubation period, cells were trypsinized and centrifuged at 1500 rpm for 10 min, the culture medium was discarded and the pellet was fixed overnight at 4 ºC with a solution of cold 70% ethanol. The cells were then centrifuged at 1500 rpm for 10 min, the pellet was resuspended in a solution of phosphate-buffered saline (PBS) containing RNAse and, after 45 min, propidium iodide was added and cells were further incubated for 1 h in the dark, at room temperature (the final concentrations of RNase and propidium iodide were 10 µg/mL and 20 µg/mL, respectively). The propidium iodide fluorescence was measured on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 nm argon-ion laser. For aggregate/debris discrimination, in addition to propidium iodide fluorescence signal heights, areas and widths were also measured. Measurements for at least 20 000 events were collected per sample. Data were analyzed using the ModFit LT 3.0. software. The experiments were carried out in duplicate for each independent experiment and the results are expressed as % of total cells.

Statistical analysis

Results are presented as the mean ± S.E.M. of the indicated number of independent experiments. Statistical significance between the different assays was determined using the one-way analysis of variance (ANOVA), followed by the Tukey post-test, for multiple comparisons. A p value <0.05 was considered statistically significant. These statistical analyses were performed using the software package GraphPad Prism 4.

Results

In order to establish the most effective GluR antagonists to pursue our work, we initially studied the effects of the NMDAR channel blockers MK-801 and memantine, and the selective NMDAR competitive antagonist APV, as well as the AMPAR antagonist NBQX, on mouse melanoma K1735-M2 cell biomass by using the SRB assay which correlates with cell number (Fig. 1). After 72 and 96 h of incubation in the presence of MK-801 and memantine, the cell biomass was decreased and significant effects were detected at 500 μM of MK-801 and at 300 μM of memantine. In contrast, the cell biomass was unaffected by 500 μM of APV or NBQX after 96 h of drug incubation (Fig. 1). Therefore, in the following experiments we used the NMDAR channel blockers MK-801 and memantine.

To elucidate the mechanism underlying the effects of the NMDAR channel blockers MK-801 and memantine, we assayed cell viability after 72 h of incubation with the drugs by using the trypan blue dye exclusion assay (Fig. 2). In agreement with the results obtained with SRB assay, NMDAR channel blockers induced a decrease in the number of viable cells, which was significant at 500 μM of MK-801 and 300 μM of memantine (Fig. 2A). Moreover, at these concentrations MK-801 and memantine did not increase the number of dead cells during the course of 72 h (Fig. 2B). The absence of an increase in the number of dead cells within 72 h of treatment with the drugs was confirmed by the LDH assay which, as the trypan blue assay, is a cytotoxicity test based on cell membrane integrity. The LDH activity did not increase in the supernatant of cells grown in the presence of MK-801 or memantine (Fig. 3A). Therefore, we investigated whether the effect of the NMDAR antagonists could be due to the inhibition of melanoma cell proliferation by means of BrdU incorporation in the DNA synthesis after a 48 h treatment with MK-801 or memantine (100-500 μM). This earlier time point was selected as the number of cells in control condition at 72 h is substantially high and could lead to absorbance values beyond the acceptable measuring range. As shown in figure 3B, both compounds significantly reduced BrdU incorporation at 300 μM. Taken together, our results indicate that the toxiceffects of the NMDAR channel blockers MK-801 and memantine on mouse melanoma K1735-M2 cells might be due to a decrease in cell proliferation.

To investigate the effect of drug combinations on mouse melanoma K1735-M2 cell proliferation, MK-801 was the compound of choice to study in association with antiestrogenic compounds, since it has been shown to be effective and safe in several animal models of cancer (Stepulak et al., 2005; North et al., 2010a, b; Song et al., 2012), whereas exposure to memantine at the concentrations used in this study compromises mitochondrial function (McAllister et al., 2008), which can lead to drug-induced tissue injury (Labbe et al., 2008).

The dose-dependent effects of antiestrogens on melanoma cells were initially evaluated by the SRB assay during a time-course experiment. The TAM active metabolite concentration of 5 μM was the lowest that induced a significant decrease in cell biomass (data not shown). Therefore, 5 μM was the selected concentration to pursue the studies aiming to assess the possible co-operative effects of a combination of antiestrogens with NMDAR antagonists on mouse melanoma K1735-M2 melanoma cell proliferation (Fig. 4).

Thus, melanoma cells were subjected to treatment with MK-801 (100 μM) and antiestrogens (5 μM), alone or in combination, over 72 h (Fig. 4). At this concentration, the antiestrogen TAM did not significantly decrease melanoma cell biomass, whereas TAM active metabolite EDX significantly reduced cell biomass to about 82 % of control, in agreement with our previous studies (Ribeiro et al., 2013). The other TAM active metabolite, OHTAM, significantly decreased cell biomass to approximately 66 % (Fig. 4). The combination of MK-801 with the antiestrogens TAM, OHTAM and EDX diminished cell biomass to approximately 46 %, 33 % and 38 % of control, respectively, which is a much stronger effect in comparison with that induced by the compounds individually. Noteworthy, MK-801 at a concentration that did not induce effects, when applied individually, co-operated with the antiestrogens to potentiate their effects (Fig. 4).

The effects of the combinations of MK-801 (100μM) with antiestrogens (5 μM) on cell viability were then quantitated at selected time points through the trypan blue dye exclusion assay (Fig. 5). As shown in figure 5A, MK-801 and TAM did not alter the number of viable cells at 72 h, in agreement with the results obtained in SRB assays (Fig. 4). However, a decrease in the number of viable cells was already observed at 48h when cells were treated with OHTAM (Fig. 5B), whereas the EDX metabolite only significantly decreased the number of viable cells at 72 h of incubation withthe

drug (Fig. 5C). The combination of any of the three antiestrogens with MK-801induced a significantly larger decrease of viable cells already observed at 48 h of incubation when compared to the compounds applied individually (Figs. 5A-5C). On the other hand, the number of dead cells after exposure to MK-801 and to the three antiestrogens, alone or in combination, did not significantly increase during the course of 72 h (Figs. 5D-5F).

The absence of an increase in the number of dead cells within 72 h of treatment with the drugs was confirmed by the LDH assay (Fig. 6). Neither the compounds individually nor their combinations increased the LDH activity in the extracellular medium, in accordance with the results obtained with the trypan blue dye exclusion assay (Fig. 5). The results obtained thus suggest that the toxic effects induced by the combined treatment of MK-801 and antiestrogens on melanoma cells may be related to a decrease in cell proliferation. Therefore, the inhibition of cell growth induced by MK-801 in association with the antiestrogens was also investigated by means of the BrdU incorporation assay (Fig. 7). While 100 μM of MK-801 by itself did not affect the BrdU incorporation in melanoma cells, 5 μM of TAM, OHTAM and EDX significantly decreased the incorporation of BrdU to 80 %, 52 % and 59 % of control, respectively. Noteworthy, the combination of MK-801 at 100 μM with the three antiestrogens TAM, OHTAM and EDX significantly decreased BrdU incorporation to 35 %, 9 % and 17 % of control, respectively, which is a much stronger effect relatively to that of the compounds individually (Fig. 7). Thus, our results showed the combinations of MK-801 and the antiestrogens might have a cytostatic effect on melanoma cells, which is more prominent when MK-801 is combined with the TAM metabolites than with the prodrug.

To confirm our hypothesis that the rate of proliferation of melanoma cells was in fact affected by the combination of MK-801 with antiestrogens, and that the reduction in BrdU signal was not a consequence of the decrease in cell number, the effect of the drugs on the cell cycle was analyzed by flow cytometry (Fig. 8). Untreated cells (control) were characterized by a long and well defined G1 peak, a slightly prominent S phase, a least prominent G2 peak and a relatively low G0/G1 fraction, which was considered as the apoptotic fraction (Fig. 8). Forty-eight hours after incubation with 100 μM of MK-801 or 5 μM of antiestrogens, the population of cells in each cell cycle phase relatively to the control condition was not changed (Fig. 8).The combination of MK-801 with the TAM active metabolites, OHTAM or EDX,

significantly increased the percentage of cells in G1 while decreasing the population of cells in the S phase, thus arresting the cell cycle in the G1 phase (Fig. 8).

Discussion

Recent studies have demonstrated that melanoma cells express NMDARs and that MK-801 inhibits their migration and proliferation (Song et al., 2012). In addition, it was reported that NMDAR and AMPAR antagonists enhance the effects of cytostatic drugs on human neuroblastoma and human rhabdomyosarcoma/medulloblastoma cell lines (Rzeski et al., 2001). Thus, we investigated whether other iGluR antagonists could also affect the proliferation of melanoma cells and the possible co-operative effects of MK-801 in combination with antiestrogenic compounds, which also decrease the growth and migration of melanoma cells (Kanter-Lewensohn et al., 2000; Matsuoka et al., 2009; Ribeiro et al., 2013). Our results show, for the first time, that the combined treatment of MK-801 with antiestrogens, and particularly with TAM active metabolites, enhances the antiproliferative action induced by the compounds individually.

The effects of GluR antagonists on mouse melanoma K1735-M2 cells were assessed by the SRB assay which showed that MK-801 and memantine reduce melanoma cell biomass (Fig. 1). On the contrary, the AMPAR and KAR antagonist NBQX, and the selective NMDAR competitive antagonist APV, which binds on the extracellular domain of the NMDAR, did not exhibit antiproliferative effects on melanoma cells even at high concentrations (Fig. 1). Although MK-801 and memantine have been traditionally considered to target theNMDAR channel, these compounds might act on other cellular targets. In fact, there is evidence that the 5-hydroxytryptamine receptor 3, the α7 and/or α4β2 nicotinic receptors and the dopamine receptors may also be involved in the biological activity of memantine (Rammes et al., 2008; Seeman et al., 2008). In addition, acute and chronic exposure to memantine has NMDAR-independent effects on the mitochondrial function, by affecting complex I and complex IV activities (McAllister et al., 2008). Likewise, MK-801 might act on the α7 and α4β2 nicotinic receptors (Briggs et al., 1996; Buisson and Bertrand, 1998) and can also modulate the dopaminergic and serotonergic system (Rao et al., 1990; Clarke and Reuben, 1995; Iravani et al., 1999). Additionally, MK-801 was shown to inhibit protein synthesis, an effect that does not appear to be related with NMDAR inhibition

(Charriaut-Marlangue et al., 1994). Thus, considering that MK-801 and memantine might interact with multiple targets, it remains unclear whether the effects on melanoma cells are mediated by NMDAR inhibition, in particular due to the lack of effect of APV. On the other hand, the absence of effect of NBQX suggests that AMPAR and KAR inhibition possibly does not affect melanoma cell viability.

The cell viability assay with trypan blue staining revealed that MK-801 and memantine decrease the number of viable cells, without inducing cell death (Fig. 2). Moreover, the evaluation of LDH activity in the supernatant of melanoma cells (Fig. 3A) and the BrdU incorporation assay pointed out that MK-801 and memantine do not induce cancer cell death, but instead they inhibit cell proliferation (Fig. 3B). Our results, obtained in mouse melanoma K1735-M2 cells, correlate with a recent study that has demonstrated that MK-801 inhibits the proliferation of the human metastatic melanoma cell line WM451, and that it can also reduce melanoma cell motility and invasion (Song et al., 2012). As metastatic malignant melanoma is largely refractory to existing therapies and has a very poor prognosis, the combined cytostatic and antimigration activity of MK-801 may suggest that it is a promising drug for melanoma treatment. Moreover, MK-801 was shown to inhibit the cell growth of other tumor cell lines and to have an antitumoral effect on animal models of melanoma (Song et al., 2012), neuroblastoma and rhabdomyosarcoma (Stepulak et al., 2005), lung (Stepulak et al., 2005; North et al., 2010a) and breast cancer (North et al., 2010b).

Considering the complex machinery involved in the onset and progression of malignant melanoma, the use of combination of drugs may provide an effective strategy to increase the therapeutic benefit (Herlyn, 2009; Ko and Fisher, 2011). Therefore, the effects of MK-801 were also investigated in combination with the antiestrogens TAM, OHTAM and EDX. Our results show, for the first time, that mouse melanoma K1735-M2 cell treatment with the NMDAR antagonist MK-801 combined with antiestrogens strongly reduces melanoma cell biomass at the concentrations used in a co-operative manner when compared with the effect induced by the compounds individually (Fig. 4). Likewise, the assessment of cell viability with trypan blue staining revealed that the combined treatment of MK-801 with antiestrogens induces a larger decrease in the number of viable cells, without increasing the number of dead cells (Fig. 5), suggesting that the observed effect of the combinations of MK-801 with antiestrogens are due to decreased cell proliferation. Indeed, the evaluation of LDH activity in the supernatant of

melanoma cells confirmed that the decrease in viable cells is not due to increased cell death (Fig. 6), whereas the BrdU incorporation assay pointed out that MK-801 and the antiestrogens inhibit cell proliferation with maximal efficacy when the drugs are used in combination (Fig. 7). Moreover, the analysis of the cell cycle revealed that the combination of MK-801 with TAM metabolites, OHTAM or EDX, induce cell cycle arrest in G1 (Fig. 8). These results are in line with other studies that have shown that GluR antagonists co-operate with other cytostatic drugs, such as cyclophosphamide, thiotepa (Rzeski et al., 2001) and docetaxel (Haas et al., 2007) enhancing the antiproliferative action. The mechanisms underlying the interaction between NMDAR antagonists and antiestrogens are not yet clarified. However, the activation of NMDAR in neurons results in the phosphorylation of extracellular regulated extracellular signal-regulated kinase (ERK) 1/2 (Kemp and McKernan, 2002; Hardingham and Bading, 2003). Accordingly, MK-801 at 250 μM decreases ERK 1/2 phosphorylation in laryngeal cancer cells (Stepulak et al., 2011), as well as in lung cancer cells (Stepulak et al., 2005). Although MK-801 at 100 μM does not affect the proliferation of melanoma cells, the combination with antiestrogens enhanced the effects induced by the compounds individually. As it seems that the mitogen-activated protein kinase (MAPK) pathway plays a pivotal role in NMDAR signaling in different types of cancer cells and TAM decreases ERK 1/2 phosphorylation in B16BL6 melanoma cells (Matuoka et al., 2009), it is possible that the effects of these drugs in combination onK1735-M2 cells involve this common pathway, which is known to play a critical in melanoma (Ko and Fisher, 2011).

According to our previous studies (Ribeiro et al., 2013), TAM active metabolites were more effective than TAM in the inhibition of the proliferation of melanoma cells, either individually or in combination. As recent studies established that the CYP2D6 phenotype is an important predictor of treatment outcome (Lammers et al., 2010) and that the coadministration of CYP2D6-inhibiting medication diminishes the treatment effect of TAM (Kelly et al., 2010), the use of TAM metabolites instead of the prodrug may increase the therapeutic benefit. Importantly, the use of MK-801 in a combined therapy might allow achieving an antitumoral effect with a lower dose than that necessary if the compound would be used in a monotherapy regimen, thus increasing the possibility of using MK-801 in a chronic treatment, without major side effects. In fact, *in vivo* studies have revealed that doses of MK-801 that were able to

slow breast (two daily doses of 0.3 mg/kg), melanoma (0.6 mg/kg every three days) and lung (up to 0.3 mg/kg) cancer progression were devoid of significant side effects (Stepulak et al., 2005; North et al., 2010a, b; Song et al., 2012). Noteworthy, the chronic exposure to MK-801 at concentrations up to 1.0 mg/kg was well tolerated by juvenile rhesus monkeys (Popke et al., 2002), suggesting that MK-801 might be suitable as a drug for cancer therapy. Nevertheless, others have found that these doses might influence rodents behavior (Gilbert, 1988; Tricklebank, 1989; Kawabe et al., 1998) and thus, the MK-801 dose and the duration of treatment necessary to achieve a maximal effect on cancer proliferation without major side effects has yet to be determined.

In conclusion, we report that the NMDAR channel blocker MK-801 and antiestrogenic compounds decrease mouse melanoma K1735-M2 cell proliferation and their therapeutic potential may be greatly enhanced when used in combination, particularly with the active metabolites of TAM.

Acknowledgments

Mariana P. C. Ribeiro is a recipient of the grant SFRH/BD/65130/2009 from the Portuguese Foundation for Science and Technology (FCT). We also wish to acknowledge the Center for Neuroscience and Cell Biology, University of Coimbra, Portugal, for financial support (PEst-C/SAU/LA0001/2013-2014).

References

Abdel-Daim M, Funasaka Y, Komoto M, Nakagawa Y, Yanagita E, Nishigori C. Pharmacogenomics of metabotropic glutamate receptor subtype 1 and in vivo malignant melanoma formation. J Dermatol 2010;37:635-46.

Briggs CA, McKenna DG. Effect of MK-801 at the human alpha 7 nicotinic acetylcholine receptor. Neuropharmacology 1996;35:407-14.

Brocke KS, Staufner C, Luksch H, Geiger KD, Stepulak A, Marzahn J, Schackert G, Temme A, Ikonomidou C. Glutamate receptors in pediatric tumors of the central nervous system.Cancer Biol Ther 2010;9:455-68.

BuissonB, Bertrand D. Open-channel blockers at the human alpha4beta2 neuronal nicotinicacetylcholine receptor. Mol Pharmacol 1998;53:555-63.

Carmo A, Carvalheiro H, Crespo I, Nunes I, Lopes MC. Effect of temozolomide on the U-118 glioma cell line. Oncol Lett 2011;2:1165-70.

Charriaut-Marlangue C, Dessi F, Ben-Ari Y. Inhibition of protein synthesis by the NMDA channel blocker MK-801.Neuroreport 1994;5:1110-2.

Clarke PB, Reuben M. Inhibition bydizocilpine (MK-801) of striatal dopamine release induced by MPTP and MPP+: possible action at the dopamine transporter. Br J Pharmacol 1995;114:315-22.

de Groot JF, Piao Y, Lu L, Fuller GN, Yung WK. Knockdown of GluR1 expression by RNA interference inhibits glioma proliferation. J Neurooncol 2008;88:121-33.

Flaherty LE, Liu PY, Mitchell MS, Fletcher WS, Walker MJ, Goodwin JW, Stephens RL, Sondak VK. The addition of tamoxifen to dacarbazine and cisplatin in metastatic malignant melanoma. A phase II trial of the Southwest Oncology Group, (SWOG-8921). Am J Clin Oncol 1996;19:108-13.

Gilbert ME. The NMDA-receptor antagonist, MK-801, suppresses limbic kindling and kindled seizures. Brain Res1988;463:90-9.

Haas HS, Pfragner R, Siegl V, Ingolic E, Heintz E, Schraml E, Schauenstein K. The non-competitive metabotropic glutamate receptor-1 antagonist CPCCOEt inhibits the in vitro growth of human melanoma.Oncol Rep 2007;17:1399-404.

Hardingham GE, Bading H. The Yin and Yang of NMDA receptor signalling.Trends Neurosci 2003;26:81-9.

Herlyn M. Driving in the melanoma landscape. Exp Dermatol 2009;18:506-8.

Holy J, Lamont G, Perkins E. Disruption of nucleocytoplasmic trafficking of cyclin D1 and topoisomerase II by sanguinarine.BMC Cell Biol 2006;7:13.

Hoogduijn MJ, Hitchcock IS, Smit NP, Gillbro JM, Schallreuter KU, Genever PG. Glutamate receptors on human melanocytes regulate the expression of MiTF. Pigment Cell Res 2006;19:58-67.

Houben R, Ortmann S, Drasche A, Troppmair J, Herold MJ, Becker JC. Proliferation arrest in B-Raf mutant melanoma cell lines upon MAPK pathway activation. J Invest Dermatol 2009;129:406-14.

Iravani MM, Muscat R, Kruk ZL. MK-801 interaction with the 5-HT transporter: a real-time study in brain slices using fast cyclic voltammetry. Synapse 1999;32:212-24.

Kanter-Lewensohn L, Girnita L, Girnita A, Dricu A, Olsson G, Leech L, Nilsson G, Hilding A, Wejde J, Brismar K, Larsson O. Tamoxifen-induced cell death in malignant melanoma cells: possible involvement of the insulin-like growth factor-1 (IGF-1) pathway. Mol Cell Endocrinol 2000;165:131-7.

Kawabe K, Yoshihara T, Ichitani Y, Iwasaki T. Intrahippocampal D-cycloserine improves MK-801-induced memory deficits: radial-arm maze performance in rats. Brain Res 1998;814:226-30.

Kelly CM, Juurlink DN, Gomes T, Duong-Hua M, Pritchard KI, Austin PC, Paszat LF. Selective serotonin reuptake inhibitors and breast cancer mortality in women receiving tamoxifen: a population based cohort study. BMJ 2010;340:c693.

Kemp JA, McKernan RM. NMDA receptor pathways as drug targets. Nat Neurosci 2002;5:Suppl:1039-42.

Kiyotani K, Mushiroda T, Nakamura Y, Zembutsu H. Pharmacogenomics of tamoxifen: roles of drug metabolizing enzymes and transporters. Drug Metab Pharmacokinet 2012;27:122-31.

Ko JM, Fisher DE. A new era: melanoma genetics and therapeutics. J Pathol 2011;223:241-50.

Labbe G, Pessayre D, Fromenty B. Drug-induced liver injury through mitochondrial dysfunction: mechanisms and detection during preclinical safety studies. Fundam Clin Pharmacol 2008;22:335-53.

Lammers LA, Mathijssen RH, van Gelder T, Bijl MJ, de Graan AJ, Seynaeve C, van Fessem MA, Berns EM, Vulto AG, van Schaik RH. The impact of CYP2D6-predicted phenotype on tamoxifen treatment outcome in patients with metastatic breast cancer. Br J Cancer 2010;103:765-71.

Lee HJ, Wall BA, Wangari-Talbot J, Shin SS, Rosenberg S, Chan JL, Namkoong J, Goydos JS, Chen S. Glutamatergic pathway targeting inmelanoma: single-agent and combinatorial therapies. Clin Cancer Res 2011;17:7080-92.

Luksch H, Uckermann O, Stepulak A, Hendruschk S, Marzahn J, Bastian S, Staufner C, Temme A, Ikonomidou C. Silencing of selected glutamate receptor subunits modulates cancer growth.Anticancer Res 2011;31:3181-92.

Marín YE, Namkoong J, Cohen-Solal K, Shin SS, Martino JJ, Oka M, Chen S. Stimulation of oncogenic metabotropic glutamate receptor 1 in melanoma cells activates ERK1/2 via PKCepsilon. Cell Signal 2006;18:1279-86.

Matsuoka H, Tsubaki M, Yamazoe Y, Ogaki M, Satou T, Itoh T, Kusunoki T, Nishida S. Tamoxifen inhibits tumor cell invasion and metastasis in mouse melanoma through suppression of PKC/MEK/ERK and PKC/PI3K/Akt pathways. Exp Cell Res 2009;315:2022-32.

McAllister J, Ghosh S, Berry D, Park M, Sadeghi S, Wang KX, Parker WD, Swerdlow RH. Effects of memantine on mitochondrial function. Biochem Pharmacol 2008;75:956-64.

McClay EF,McClay ME, Jones JA, Winski PJ, Christen RD, Howell SB, Hall PD. A phase I and pharmacokinetic study of high dose tamoxifen and weekly cisplatin in patients with metastatic melanoma. Cancer 1997;79:1037-43.

Namkoong J, Shin SS, Lee HJ, Marín YE, Wall BA, Goydos JS, Chen S. Metabotropic glutamate receptor 1 and glutamate signaling in human melanoma. Cancer Res 2007;67:2298-305.

North WG, Fay MJ, Du J, Cleary M, Gallagher JD, McCann FV. Presence of functional NMDA receptors in a human neuroblastoma cell line. Mol Chem Neuropathol 1997;30:77-94.

North WG, Gao G, Jensen A, Memoli VA, Du J. NMDA receptors are expressed by small-cell lung cancer and are potential targets for effective treatment. Clin Pharmacol 2010a;2:31-40.

North WG, Gao G, Memoli VA, Pang RH, Lynch L. Breast cancer expresses functional NMDA receptors. Breast Cancer Res Treat 2010b;122:307-14.

Popke EJ, Patton R, Newport GD, Rushing LG, Fogle CM, Allen RR, Pearson EC, Hammond TG, Paule MG. Assessing the potential toxicity of MK-801 and remacemide: chronic exposure in juvenile rhesus monkeys. Neurotoxicol Teratol 2002;24:193-207.

Rammes G, Danysz W, Parsons CG. Pharmacodynamics of memantine: an update. Curr Neuropharmacol 2008;6:55-78.

Rao TS, Kim HS, Lehmann J, Martin LL, Wood PL. Interactions of phencyclidine receptor agonist MK-801 with dopaminergic system: regional studies in the rat. J Neurochem 1990;54:1157-62.

Ribeiro MP, Silva FS, Paixão J, Santos AE, Custódio JB. The combination of the antiestrogen endoxifen with all-*trans*-retinoic acid has anti-proliferative and anti-migration effects on melanoma cells without inducing significant toxicity in non-neoplasic cells. Eur J Pharmacol 2013;715:354-362.

Rzeski W, Turski L, Ikonomidou C. Glutamate antagonists limit tumor growth. Proc Natl Acad Sci USA. 2001;98:6372-7.

Schroth W, Goetz MP, Hamann U, Fasching PA, Schmidt M, Winter S, Fritz P, Simon W, Suman VJ, Ames MM, Safgren SL, Kuffel MJ, Ulmer HU, Boländer J, Strick R, Beckmann MW, Koelbl H, Weinshilboum RM, Ingle JN, Eichelbaum M, Schwab M, Brauch H. Association between CYP2D6 polymorphisms and outcomes

among women with early stage breast cancer treated with tamoxifen. JAMA 2009;302:1429-36.
Seeman P, Caruso C, Lasaga M. Memantine agonist action at dopamine D2High receptors. Synapse 2008;62:149-53.
Song Z, He CD, Liu J, Sun C, Lu P, Li L, Gao L, Zhang Y, Xu Y, Shan L, Liu Y, Zou W, Zhang Y, Gao H, Gao W. Blocking glutamate-mediated signalling inhibits human melanoma growth and migration. Exp Dermatol 2012;21:926-31.
Stepulak A, Sifringer M, Rzeski W, Endesfelder S, Gratopp A, Pohl EE, Bittigau P, Felderhoff-Mueser U, Kaindl AM, Bührer C, Hansen HH, Stryjecka-Zimmer M, Turski L, Ikonomidou C. NMDA antagonist inhibits the extracellular signal-regulated kinase pathway and suppresses cancer growth. Proc Natl Acad Sci USA. 2005;102:15605-10.
Stepulak A, Sifringer M, Rzeski W, Brocke K, Gratopp A, Pohl EE, Turski L, Ikonomidou C. AMPA antagonists inhibit the extracellular signal regulated kinase pathway and suppress lung cancer growth. Cancer Biol Ther 2007;6:1908-15.
Stepulak A, Luksch H, Gebhardt C, Uckermann O, Marzahn J, Sifringer M, Rzeski W, Staufner C, Brocke KS, Turski L, Ikonomidou C. Expression of glutamate receptor subunits in human cancers. Histochem Cell Biol 2009;132:435-45.
Stepulak A, Luksch H, Uckermann O, Sifringer M, Rzeski W, Polberg K, Kupisz K, Klatka J, Kielbus M, Grabarska A, Marzahn J, Turski L, Ikonomidou C. Glutamate receptors in laryngeal cancer cells. Anticancer Res 2011;31:565-73.
Teh JL, ChenS. Glutamatergic signaling in cellular transformation. Pigment Cell Melanoma Res 2012;25:331-42.
Tricklebank MD, Singh L, Oles RJ, Preston C, Iversen SD. The behavioural effects of MK-801: a comparison with antagonists acting non-competitively and competitively at the NMDA receptor. Eur J Pharmacol 1989;167:127-35.
Vieira M, Fernandes J, Burgeiro A, Thomas GM, Huganir RL, Duarte CB, Carvalho AL,Santos AE. Excitotoxicity through Ca2+-permeable AMPA receptors requires Ca2+-dependent JNK activation. Neurobiol Dis 2010;40:645-55.

Figure legends

Fig. 1. Effects of GluR antagonists on mouse melanoma K1735-M2 cell biomass. Cells were incubated in the absence (control) or in the presence of MK-801 (100-500 μM), memantine (100-500 μM), APV (500 μM) and NBQX (500 μM). At 72 and 96 h, melanoma cell biomass was evaluated by the SRB assay. For that purpose, the cells were fixed with absolute methanol containing 1 % acetic acid and incubated with SRB solution at 37 °C for 1 h. Afterwards, the plates were rinsed and the bound dye eluted with Tris buffer and the absorbance was measured at 540 nm. Bars represent the mean ± S.E.M. of four independent experiments performed in triplicates. *** $p<0.001$ vs the respective time point control, One-way ANOVA followed by Tukey post-test.

Fig. 2. Cell viability of melanoma cells treated with the NMDAR channel blockers MK-801 and memantine. Mouse melanoma K1735-M2 cells were grown in the absence (0) or in the presence of 100-500 μM of MK-801 or memantine for 72 h and the number of viable and dead cells was determined by the trypan blue dye exclusion assay. After the incubation period, cells were trypsinized, centrifuged, treated with 0.4 % trypan blue and counted in a hemocytometer under a transmitted light microscope. The number of trypan blue-negative (viable) cells and trypan blue-positive (dead) cells is presented in the graphs A and B, respectively. Data represent the mean ± S.E.M. of four independent experiments. *** $p<0.001$, * $p<0.05$ vs control.

Fig. 3. The NMDAR channel blockers MK-801 and memantine do not induce cell death (A) and decrease cell proliferation (B). (A) Mouse melanoma K1735-M2 cells were grown in the absence (control) or in the presence of 100-500 μM of MK-801 or memantine. Cell death was assessed by measuring LDH activity in the supernatant of damaged cells after 72 h in culture. Bars represent the mean ± S.E.M. of three independent experiments performed in duplicates. The statistical analysis was performed by One-way ANOVA followed by Tukey post-test. (B) Cells were grown for 48 h in the absence (control) or in the presence of 100-500 μM of MK-801 or memantine and then cell proliferation was assessed by the BrdU incorporation assay as described in the Materials and methods section. Bars represent the mean ± S.E.M. of four independent experiments performed in triplicates. *** $p<0.001$, ** $p<0.01$vs control, One-way ANOVA followed by Tukey post-test.

Fig. 4. The combined treatment of MK-801 with antiestrogens potentiates the decrease in mouse melanoma K1735-M2 cell biomass induced by the compounds individually. Melanoma cells were grown in the absence (control) or in the presence of 100 μM of MK-801, 5 μM of the antiestrogens TAM, OHTAM and EDX, alone or in combination. The melanoma cell biomass was evaluated by the SRB assay after 72 h of incubation. Bars represent the mean ± S.E.M. of six independent experiments performed in triplicates. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ vs control. +++ $p<0.001$ vs MK-801. ### $p<0.001$, # $p<0.05$ vs antiestrogen, One-way ANOVA followed by Tukey post-test.

Fig. 5. Cell viability of mouse melanoma K1735-M2 cells treated with MK-801 and the antiestrogens.Melanoma cells were grown in the absence (control) or in the presence of 100 μM of MK-801 and 5 μM of the antiestrogens TAM (A, D), OHTAM (B, E) and EDX (C, F), alone or in combination, and cell viability was assessed by the trypan blue dye exclusion assay as described in the Materials and methods section at 24 h, 48 h and 72 h. The graphs present the number of viable (A, B, C) and dead (D, E, F) cells. Data represent the mean ± S.E.M. of six independent experiments. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ vs the respective time point control. +++ $p<0.001$, ++ $p<0.01$ vs MK-801 at the respective time point. ### $p<0.001$, ## $p<0.01$, # $p<0.05$ vs the antiestrogen at the respective time point, One-way ANOVA followed by Tukey post-test.

Fig. 6. The combination of MK-801 with the antiestrogens does not induce melanoma cell death. Mouse melanoma K1735-M2 cells were grown in the absence (control) or in the presence of 100 μM of MK-801, 5 μM of the antiestrogens TAM, OHTAM and EDX, alone or in combination. Cell death was assessed by measuring LDH release from damaged cells, after 72 h in culture. Bars represent the mean ± S.E.M. of four independent experiments performed in duplicates.

Fig. 7. Mouse melanoma K1735-M2 cell treatment with the combination of MK-801 and the antiestrogens reduces cell proliferation.Cells were grown in the absence (control) or in the presence of 100 μM of MK-801, 5 μM of the antiestrogens TAM, OHTAM and EDX, alone or in combination, for 48 h, and then cell proliferation was assessed by the BrdU incorporation assay as described in the Materials and methods section. Bars represent the mean ± S.E.M. of four independent experiments performed in triplicates. *** $p<0.001$, * $p<0.05$ vs control. +++ $p<0.001$ vs MK-801. ### $p<0.001$, ## $p<0.01$ vs antiestrogen, One-way ANOVA followed by Tukey post-test.

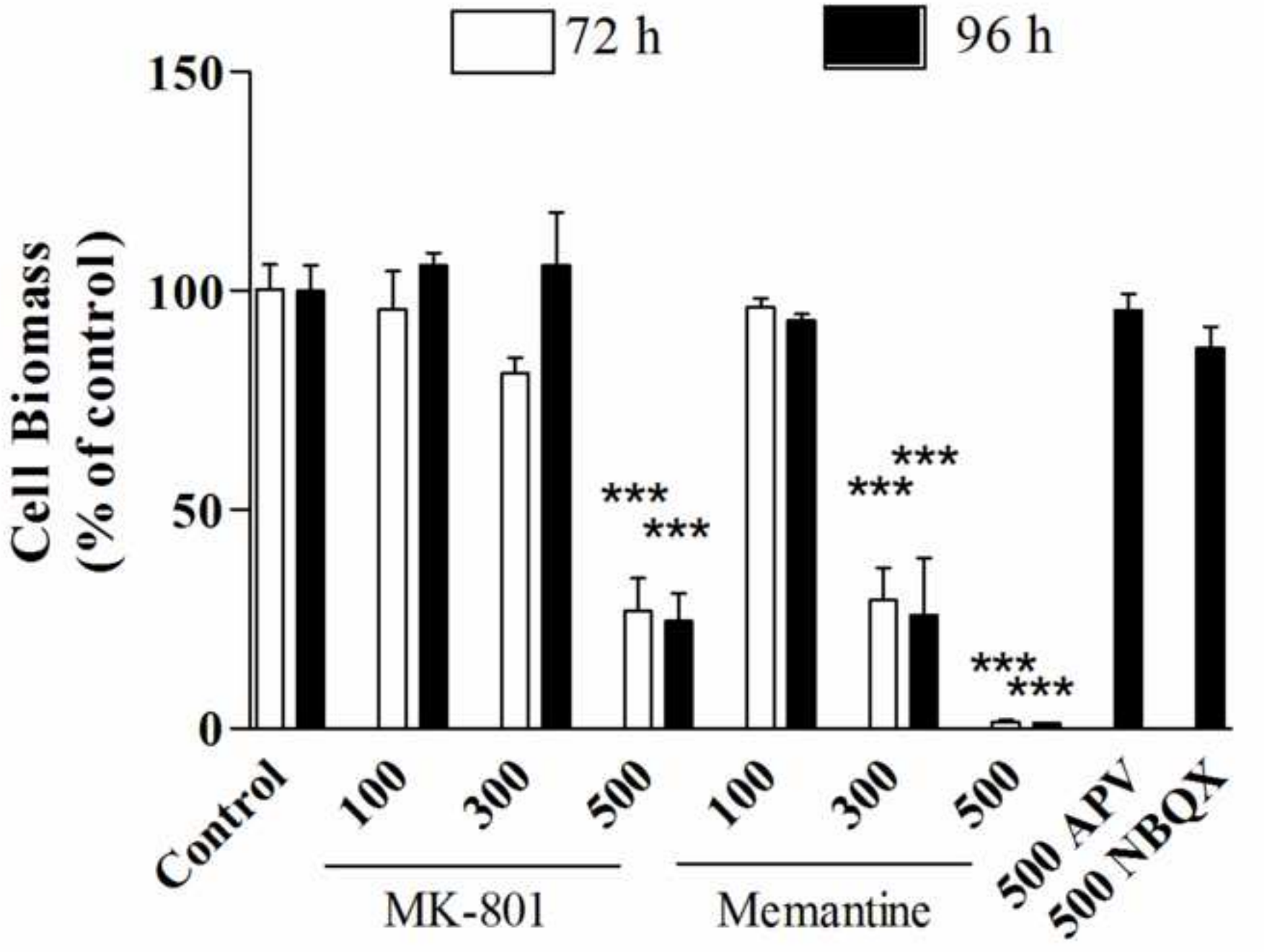
Fig. 8. Melanoma cell treatment with MK-801 and TAM metabolites blocks cell cycle progression in G1. Mouse melanoma K1735-M2 cells were grown in the absence (control) or in the presence of MK-801 (100 μM), 5 μM of the antiestrogens TAM, OHTAM and EDX, alone or in combination, for 48 h. Cell cycle distribution was evaluated by flow cytometry analysis of the DNA content labeled with propidium iodide. Data are the mean ± S.E.M. of three independent experiments performed in

duplicates. A total of 20 000 events were analyzed for each experiment. ** $p<0.01$, * $p<0.05$ vs control. + $p<0.05$ vs MK-801. ## $p<0.01$, # $p<0.05$ vs the antiestrogen, One-way ANOVA followed by Tukey post-test.

Highlights

- MK-801 and memantine decrease melanoma cell proliferation.
- The combination of MK-801 with antiestrogens inhibits melanoma cell proliferation.
- These combinations greatly enhance the effects of the compounds individually.
- MK-801 combined with tamoxifen active metabolites induces cell cycle arrest in G1.
- The combination of MK-801 and antiestrogens is an innovative strategy for melanoma.

Figure 1



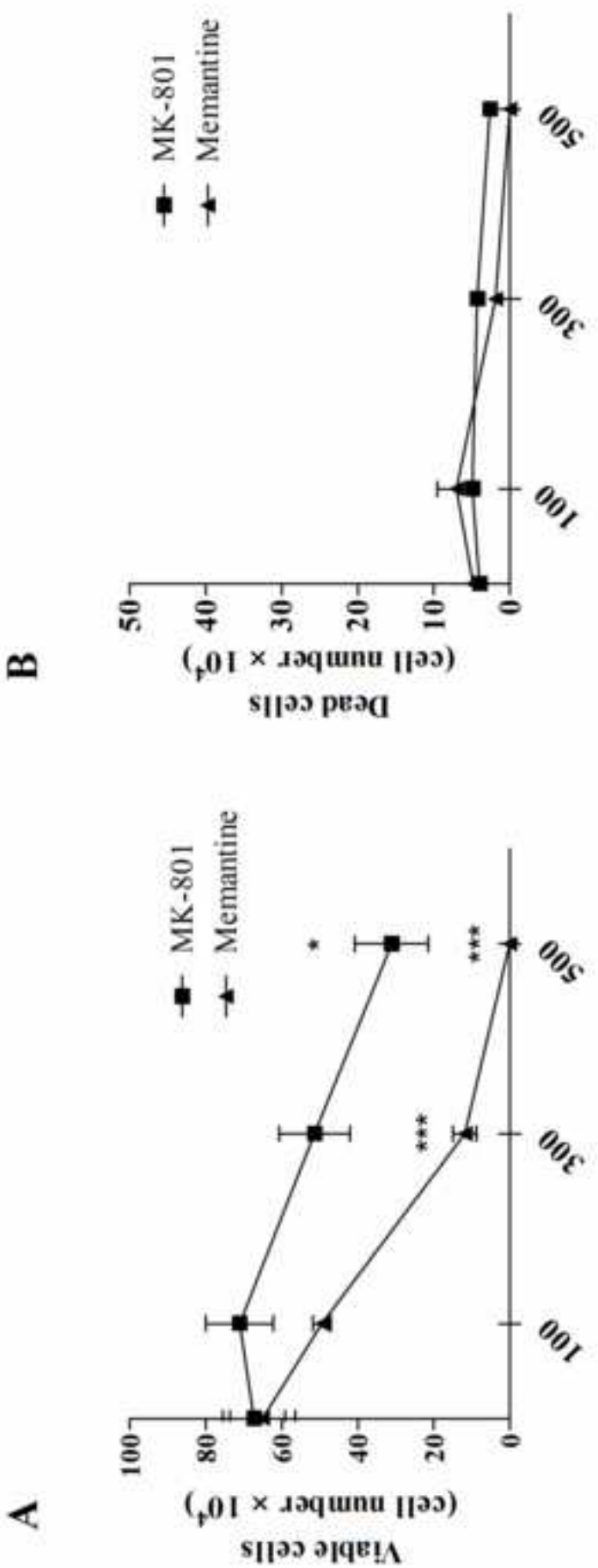


Figure 2

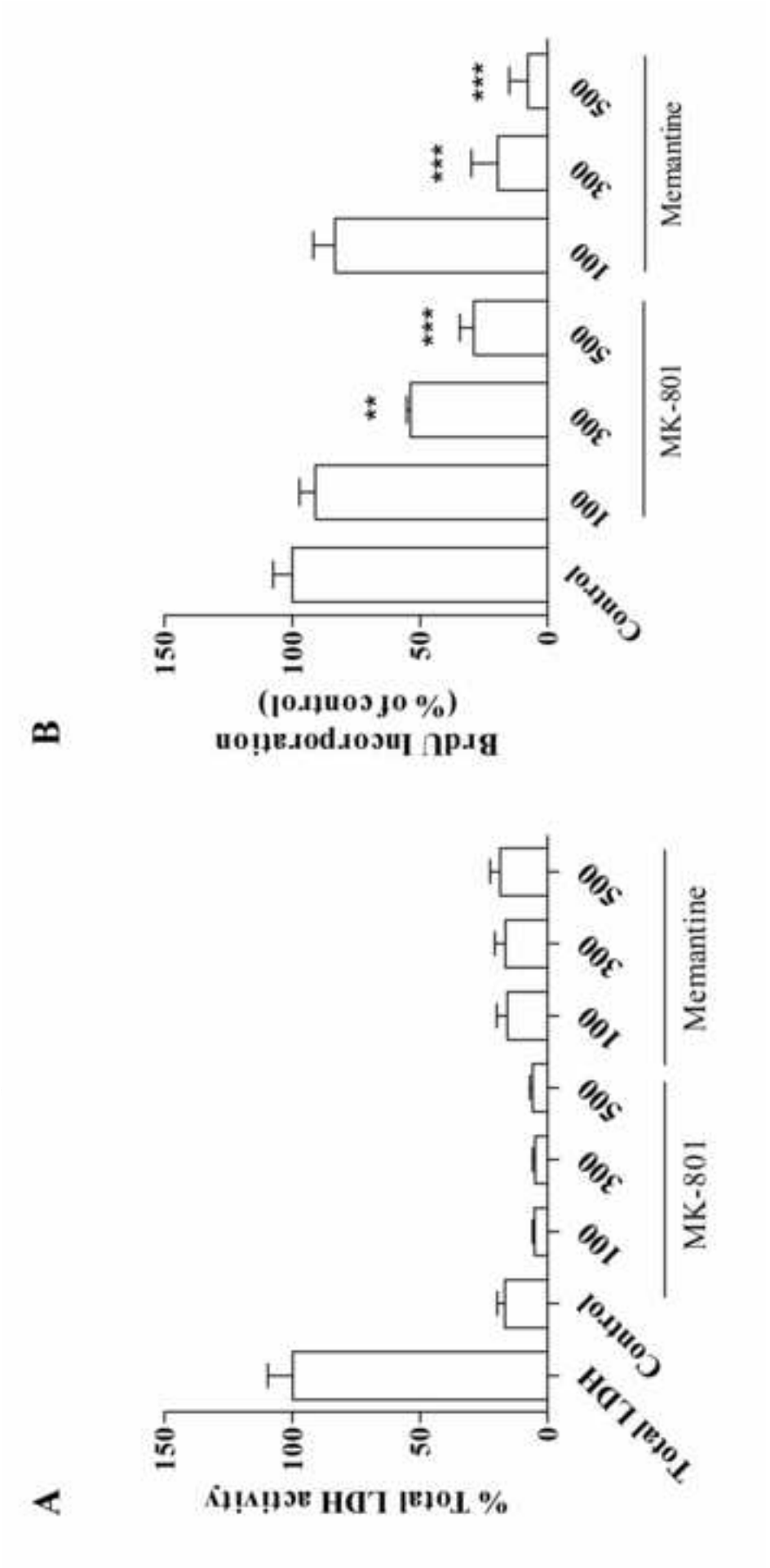


Figure 3

Figure 4

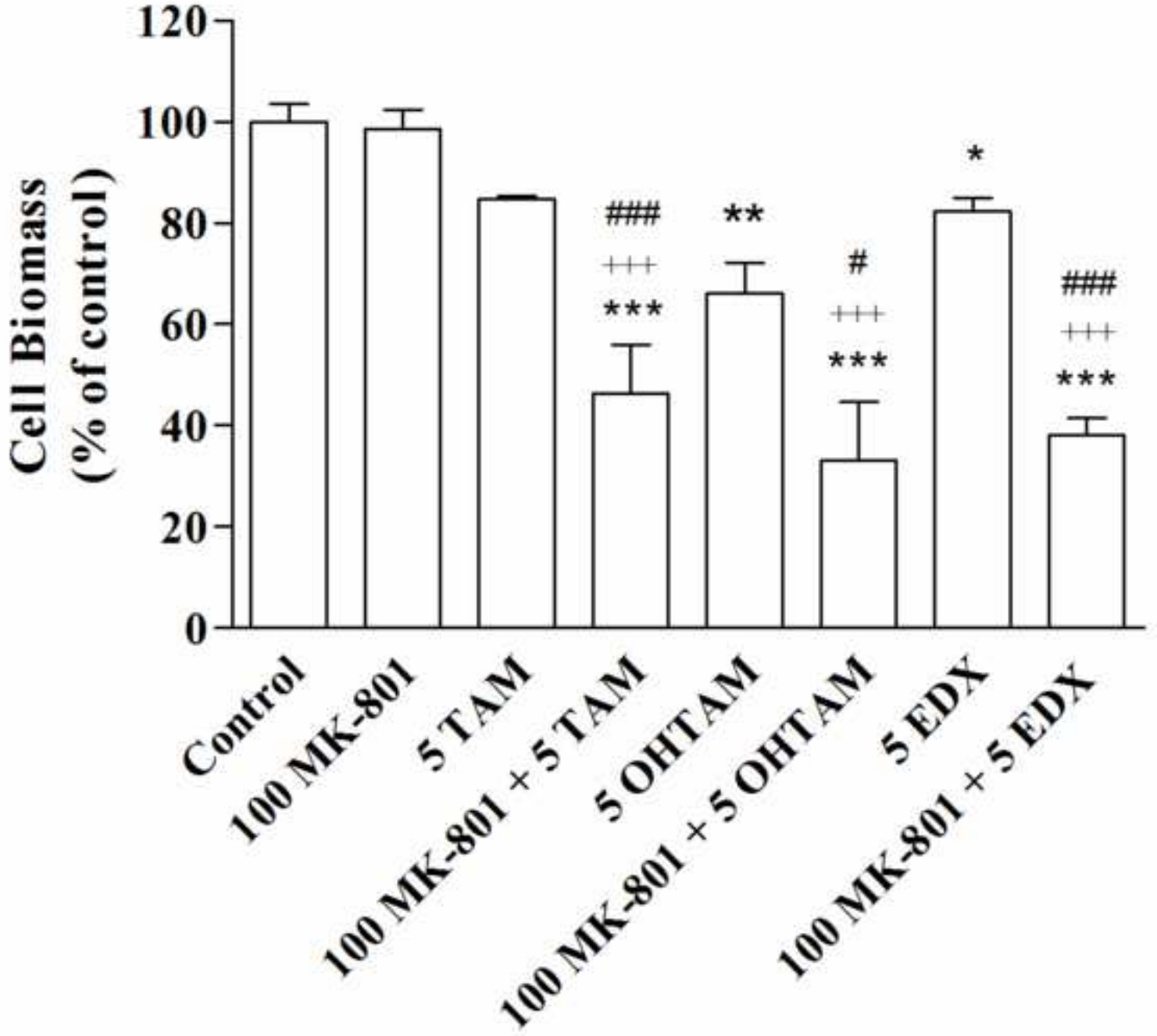
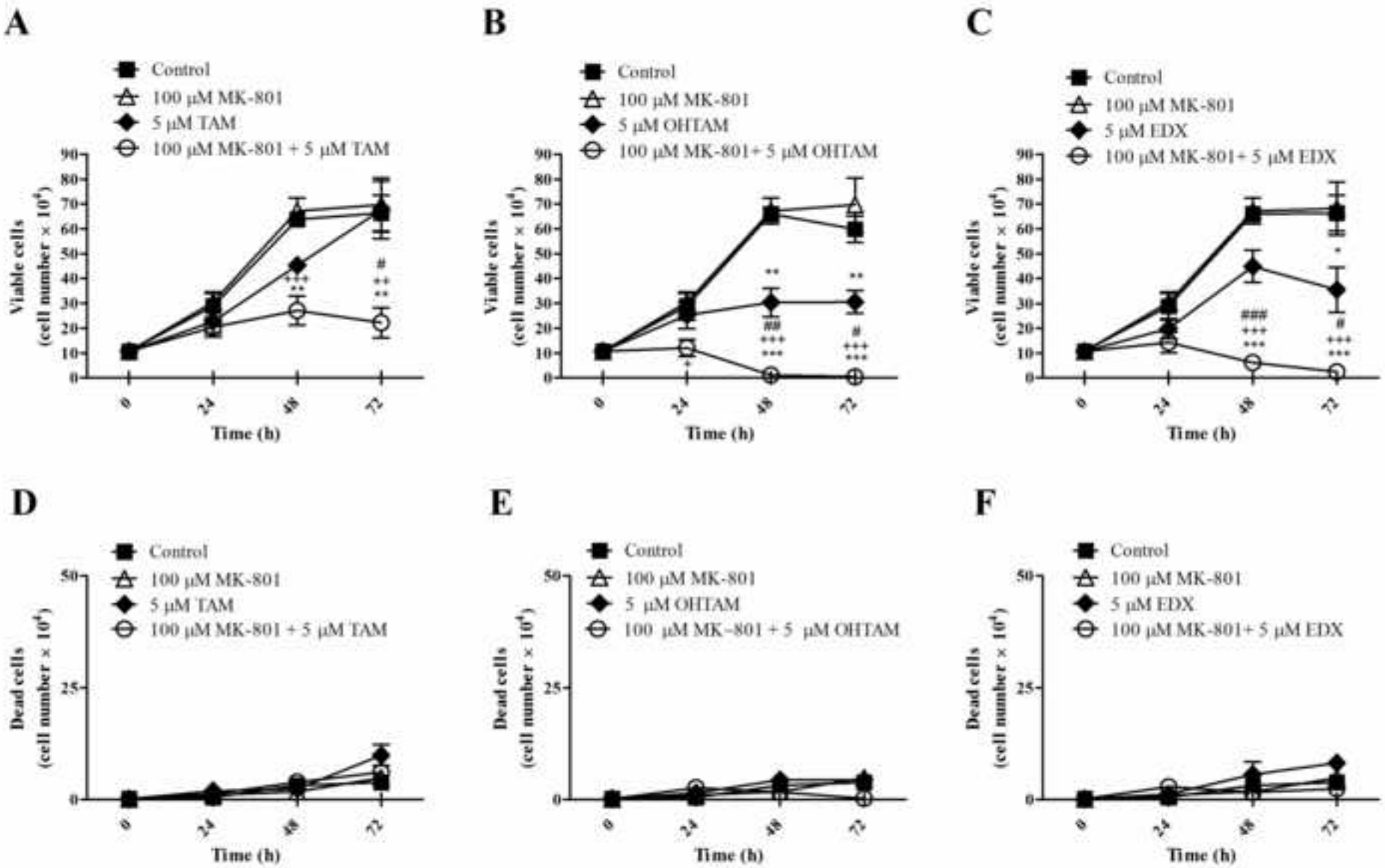


Figure 5



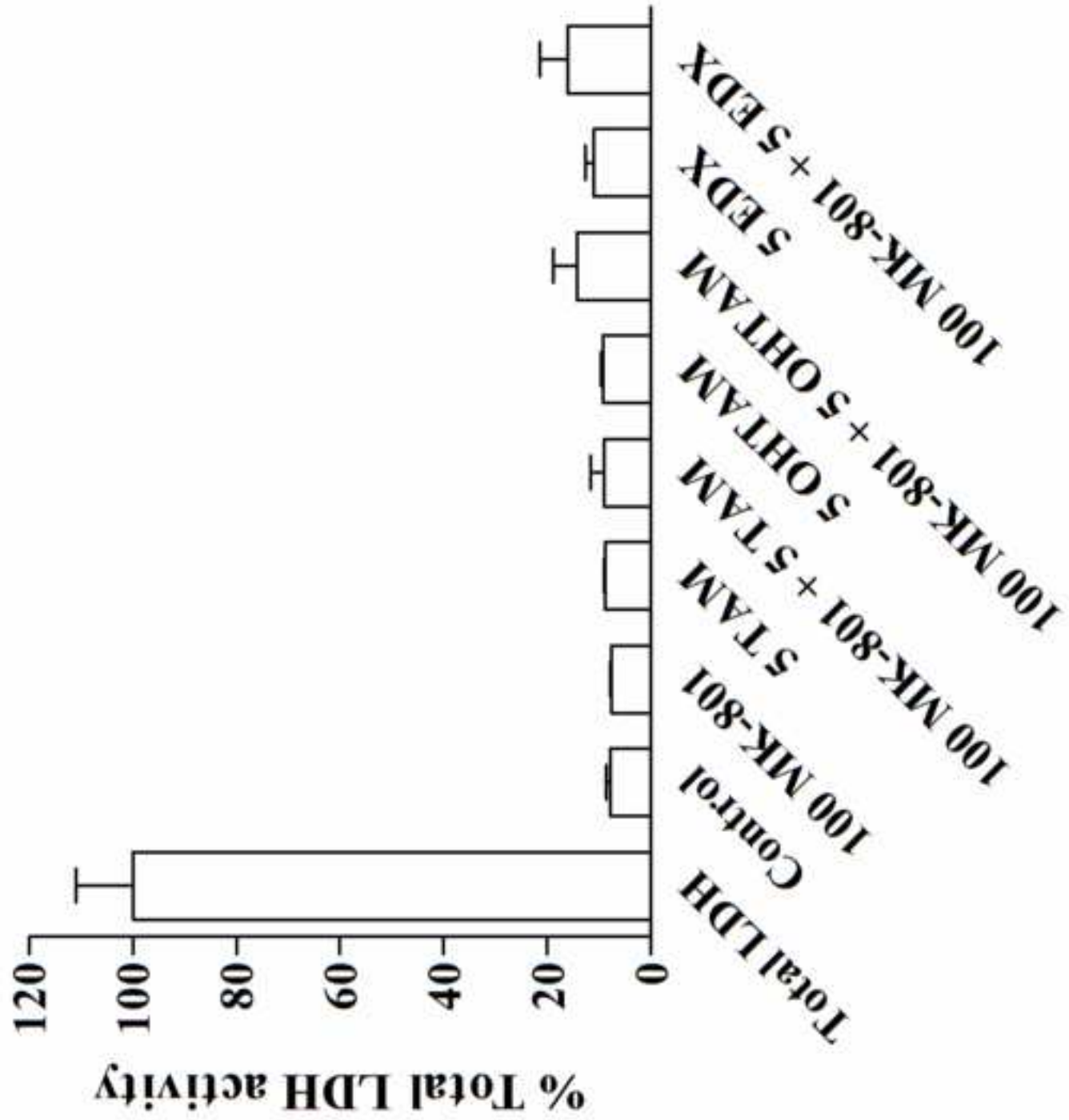

% Total LDH activity
120
100
80
60
40
20
0
Total LDH
Control
100 MK-801
5 TAM
100 MK-801 + 5 TAM
5 OHTAM
100 MK-801 + 5 OHTAM
5 EDX
100 MK-801 + 5 EDX

Figure 6

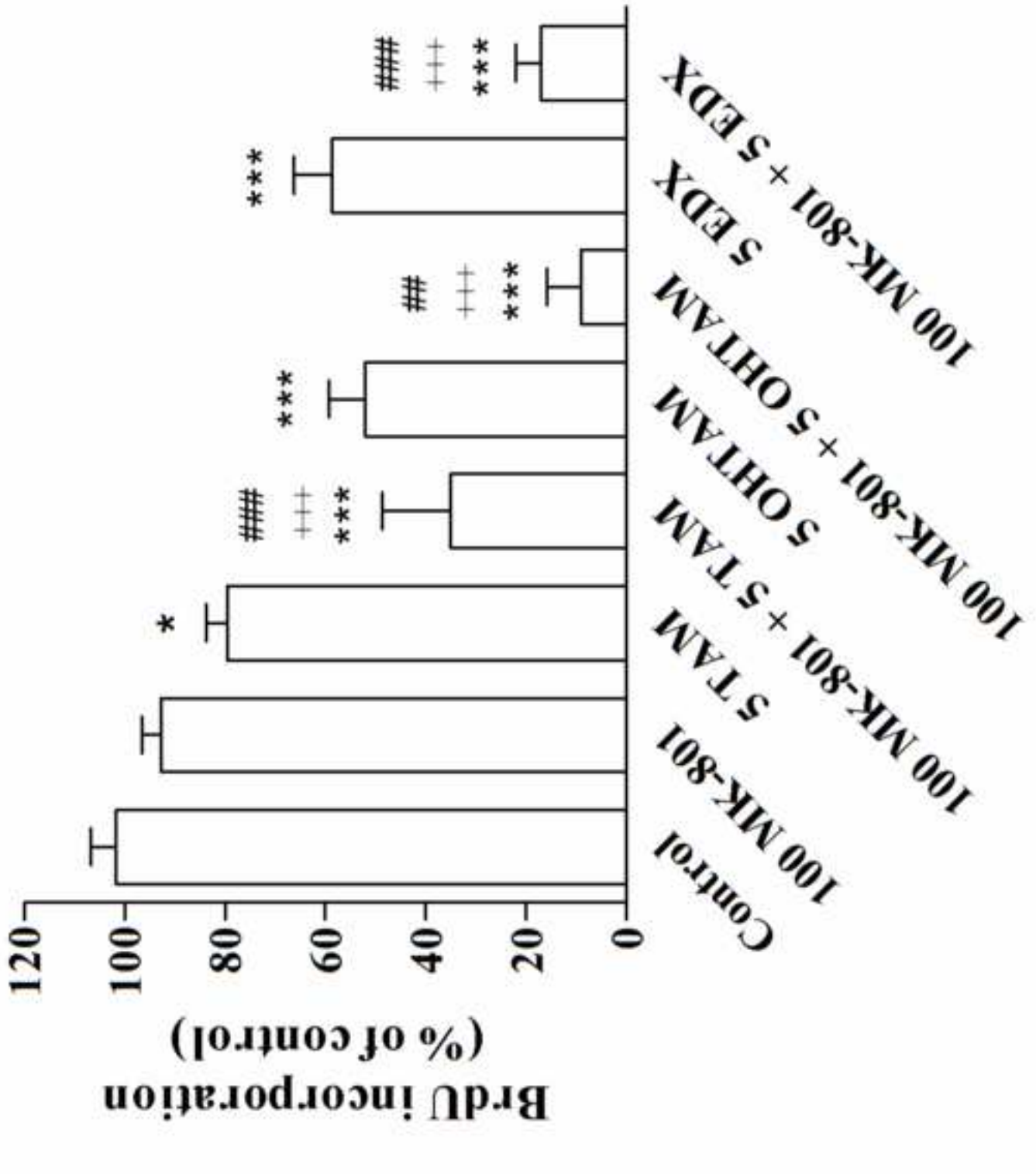

BrdU incorporation
(% of control)
120
100
80
60
40
20
0
Control
100 MK-801
5 TAM
100 MK-801 + 5 TAM
5 OHTAM
100 MK-801 + 5 OHTAM
5 EDX
100 MK-801 + 5 EDX
*
****
‡‡‡‡
####
****
‡‡‡‡
##
****
****
‡‡‡‡
####

Figure 7

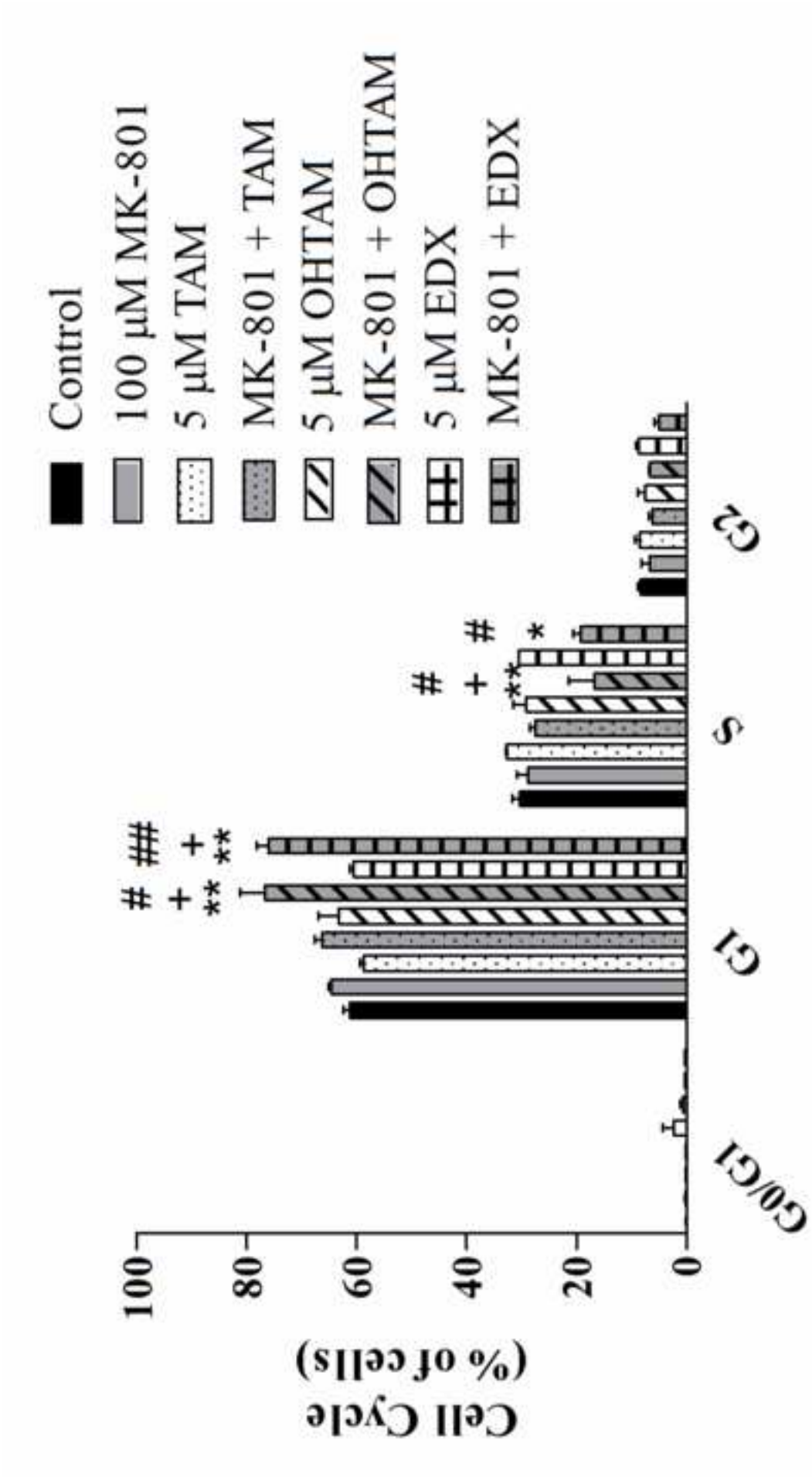

Control
100 μM MK-801
5 μM TAM
MK-801 + TAM
5 μM OHTAM
MK-801 + OHTAM
5 μM EDX
MK-801 + EDX
Cell Cycle
(% of cells)
100
80
60
40
20
0
G0/G1
S
G2
#
##
+
++
**
**

Figure 8