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2-Methoxyestradiol induces morpho-functional changes and impairs the microtubular system in mouse neuroblastoma and rat glioma cells

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ABSTRACT

2-Methoxyestradiol (2ME), a metabolite deriving from 17-β estradiol, is a well-established antiangiogenic, apoptotic and antiproliferative agent in cell cultures and animal models. 2ME may also exert its cytotoxic activity by interacting with tubulin and by causing an impairment of the microtubular system. The aim of this study was to investigate the relative effectiveness of 2ME on mouse neuroblastoma (C1300) and rat glioma (C6) cell lines in inducing morpho-functional changes and alteration of the microtubular system physiology. Cells, cultured in a medium supplemented with increasing 2ME micromolar concentrations, were submitted to morphological investigations, MTT assay and western blot analysis. 2ME-exposed cell lines displayed in comparison with control cells, morpho-functional changes such as reduction in cell number, a globular/shrunken shape, retraction or absence of cytoplasmic processes, inhibition of cell growth and cell decreased viability. Interestingly, all changes detected were more evident in C1300 cells than in C6 cells. Western blot analysis showed that the total and the tyrosinated α-tubulin expression was reduced more intensely in the C1300 than in C6 cells; whereas the acetylated α-tubulin expression did not significantly decrease in either cell lines. Results demonstrate that 2ME is more effective in neural cells than in glial cells. The alteration of total and tyrosinated α-tubulin expression suggests that 2ME effectiveness could be strictly related to an impairment of microtubule system physiology resulting in morpho-functional changes, block of mitosis and cell death.

Key words

2-Methoxyestradiol • C1300 Neuroblastoma cells • C6 Glioma cells • Tubulin

Introduction

2-Methoxyestradiol (2ME) is an active, endogenous metabolite of 17β-estradiol formed by sequential hydroxylation in position 2 (Brueggemeier and Singh, 1989) which has been developed as a novel antitumoral agent. This drug has a low binding affinity for oestrogen receptors (ERs) α and β consistent with its low estrogenic activity thus, it is still a matter of debate as to whether 2ME biological activity is mediated by ERs (Martucci and Fishman, 1979; LaVallee et al., 2002; Banerjee et al., 2003). 2ME exerts a strong antiangiogenic activity, induces apoptosis and inhibits cell proliferation at micromolar concentrations both in cell cultures and animal models. The body of evidence suggests that 2ME cytoxicity is due to several mechanisms including effects on superoxide dismutase (Huang et al., 2000; Kachadourian et al., 2001), phosphorylation of Bcl-2 (Bu et al., 2006), up-regulation of p53 (Seegers et al., 1997; Shimada et al., 2003), up-regulation of death receptors 5 (LaVallee et al., 2003) and dysregulation

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of hypoxia-inducible factor-1 (HIF) (Mabjeesh et al., 2003). 2ME, like colchicine, depolymerizes microtubules by binding to tubulin. Specifically, 2ME competes for the colchicine binding-site and this activity results in the inhibition of tubulin nucleation or propagation (D'Amato et al., 1994; Hamel et al., 1996). Further studies have shown the capability of 2ME to cause atypical polymerization of the mitotic spindle (Tinley et al., 2003) and to produce an abnormal methaphase in a broad range of cancer cell lines (Seegers et al., 1997). Moreover, some studies have proved that 2ME is able to exert its cytotoxicity by causing an impairment of the microtubular system rather than by determining microtubular depolymerization. Indeed, 2ME has been found to be more effective in high-proliferative dividing cells than in slow-growing not-proliferative cells (Seegers et al., 1997; Brueggemeier et al., 2001). Kamath and co-workers have also demonstrated that the disruption of tubulin dynamics alters microtubule growth parameters, increases the tubulin *steady-state* and blocks chromosome progression (Kamath et al., 2006). Alteration of microtubular system physiology is mainly related to the dynamic status of the microtubules (Rochlin et al., 1996). Microtubules originate from the polymerization of tubulin proteins (heterodimers constituted of α- and β- subunits) and exhibit a great heterogeneity of components. Microtubules are functionally modified into several isoforms by post-translational modifications that affect the carboxy-terminal domain of the αβ-tubulin heterodimer (MacRae, 1997). Among these post-translational modifications, the cyclic tyrosination/detyrosination of the C-terminus and the acetylation at Lys 40, have been found to be related to microtubular dynamics (MacRae, 1997). Dynamic microtubules, involved in cellular processes such as mitosis/meiosis, maintenance of cell shape, elongation of cell processes and cellular differentiation, are tyrosinated (Gundersen et al., 1987). However, the acetylated α-tubulin (acet-tub) is an isoform considered typical of microtubule stabilization since it is expressed by non actively-dividing cells or cells treated with a microtubular stabilizing drug such as nocodazole (Rochlin et al., 1996). Thus, the tyr-tub and acet-tub isoforms are currently used as markers of the dynamic or stable form of microtubules, respectively (Gundersen et al., 1987; Rochlin et al., 1996; Contin and Arce, 2000).

In the present study the relative effectiveness of 2ME on neoplastic cells of neural and glial origin has been evaluated. Despite their phenotypic features C1300 and C6 cells shown neuron-like and glial-like properties. Undifferentiated mouse neuroblastoma (clone C1300) and rat glioma (clone C6) were exposed to 2ME micromolar concentrations in order to assess possible morphological and functional alterations (reduction in cell number, globular/shrunken shape, retraction or absence of cytoplasmic processes, inhibition of cell growth and decreased cell viability) induced by the drug-exposure. In addition, in order to investigate the potential impairment of the microtubular system, the expression of acet-tub, tyr-tub and total α-tubulin expression has been investigated by western blot.

Materials and methods

Undifferentiated C1300 and C6 cell lines (*American Type Culture Collection*, Rockville, MD, USA) were used. The specificity of the two cell lines was ascertained by immunodetecting the neuron-specific class β-III tubulin in C1300 and glial fibrillary protein (GFAP) in C6, with the immunofluorescence technique (data not shown). At passage 60 cells were cultured in a basal phenol-red-free RPMI-1640 medium supplemented with 10% heat inactivated dextran-coated charcoal-stripped newborn calf serum, 2 μM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin sulphate. Cells were seeded in 75 cm^2 flasks with a basal medium at a concentration of 5.6 x 10^5 per ml; cells were grown at 37°C in a 5% CO_2 humidified atmosphere. Only a basal medium was used for the control cells; the amounts of 2ME (*Sigma*, Saint Louis, MO, USA) were added to the basal medium. The final 2ME micromolar concentrations (0.1 μM, 1 μM, 5 μM) and exposure-time were chosen taking into account analogous *in vitro* experiments performed on neoplastic cells of neural (Nakagawa-Yagi et al., 1996), glial origin (Lis et al., 2004; Braeuninger et al., 2005; Chamaon et al., 2005) and other cancer cell lines (Seegers et al., 1989; Seegers et al., 1997; Kamath et al., 2006).
2ME micromolar concentrations were serially diluted from a stock solution containing 20 mg of 2ME dissolved in 1 ml DMSO (0.00001% v/v). To check the possible toxic effect of DMSO on cells, a fur-

ther control was set by incubating cells in a basal medium containing DMSO at the same concentration applied to dissolve 2ME (DMSO control cells or, briefly, control cells). Cells were treated with 2ME for 5 days; after 3 days the culture medium was replaced by fresh medium. These procedures were carried out in accordance with general cell culture procedures; moreover, the exposure time was chosen taking into account analogous *in vitro* studies (Seegers et al., 1989; Nakagawa-Yagi et al., 1996; Seegers et al., 1997; Lis et al., 2004; Braeuninger et al., 2005; Chamaon et al., 2005; Kamath et al., 2006) and our experimental experience.

C1300, C6 monolayers were daily observed in the living state under phase-contrast optics (PHACO) at a magnification of 20X with an inverted microscope (*Leitz*, DMIL), photographed by a digital camera and processed by western blot in accordance with a validated protocol. Briefly, cells were suspended in a stabilizing solution (5 μM TRIS HCl pH 8.0, 2 μM EGTA, 0.1 μM phenylmethylsulfonyl fluoride) and supplemented with protease inhibitors (Complete-mini, *Roche*, Basel, Switzerland). Cells were mechanically homogenized and sonicated. The protein content was determined (DC Protein Assay, *BioRad*, Hercules, CA, USA) and aliquots of 15 μg proteins each, were fractionated in SDS-PAGE 12% gel and finally transferred onto nitrocellulose membranes. Non-specific protein binding was blocked by incubating membranes with 0.1% Tween 20 and 5% skim milk in PBS. Nitrocellulose membranes were incubated with primary monoclonal antibodies against total α-tubulin (clone DM1A, *Sigma*), acetylated-α-tubulin (clone 6-11B-1, *Sigma*) and tyrosinated α-tubulin (clone TUB-1A2, *Sigma*), overnight at 4°C at 1:500, 1:500, 1:1.000 dilution, respectively. An anti-mouse IgG-alkaline phosphatase-conjugated antibody (Anti-mouse Ig-AP, Fab fragment, *Roche*) was used at 1:1.000 dilution for 1 h at room temperature. The immunoreactive bands were visualized by incubating the membrane with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, *Roche*) and were heat fixed. Signals were quantified by Scion Image software. The anti α-tubulin antibody visualized the α-tubulin subunits as a single band located approximately at 55 kDa.

Furthermore, a cell growth assay was performed in 96-well microtiter plates containing 50 x 10^3 cells in 200 μl medium per well. The cells underwent all protocols described above then were incubated in 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (MTT) (MTT Cell Titer96®, *Promega*, Madison, WI, USA) for 1 h. MTT was metabolically reduced by proliferative cells into a coloured formazan product that is soluble in tissue culture medium. Absorption values were read by setting an automatic microtiter reader at 492 μm (Uniskan II, Labsystem. Helsinki, Finland). This method correlates absorbance from the amount of coloured product to the number of vital and proliferative cells (Cory et al., 1991). Results were calculated as a percentage of control cells. IC_{50} was also calculated as the concentration of 2ME that produces 50% cell growth inhibition, i.e., reduction in absorbance. A cell viability assay was performed as well: monolayers were stained with bisbenzimide (Hoechst H33342, *Sigma*) and propidium iodide (PI) (*Sigma*). Cells were cultured in chamber slides according to the protocol above indicated. Vital cells were stained with Hoechst 33342 (*Sigma*) while the dead cells were stained with PI (*Sigma*). Cell death rate was estimated as deads cell *vs.* total cell number x 100 and the intra-observer and inter-observer variability were each < 2%.

All experimental data were expressed as means ± S.E.M. of 6 experiments, data were tested by one-way ANOVA or Student's *t* test and a probability of 0.05 was considered significant.

Results

When observed under contrast phase optics, 2ME-exposed cells displayed morphological alterations. Concentration ranging from 0.1 μM in C1300 and 1 μM in C6 cells respectively, 2ME determined a decrease in cell number in comparison with control cells. Cell cultures showed a flattened/shrunken shape as well; in addition, at the highest concentration tested (5 μM) most of cells were detached from the dishes and retraction or absence of cytoplasmic processes was detected (Fig. 1). All these alterations were more evident in C1300 than in C6 cells. Moreover, the viability assay (Hoechst H33342/PI staining) showed that cell death was greater in C1300 than in C6 cells. The dead cell percentage (PI red-stained cells) was about 60-90% in C1300 cells and 30-50% in C6 cells at 1 μM and 5 μM, respectively. Control monolayers (ctrl) and 0.1 μM 2ME-exposed

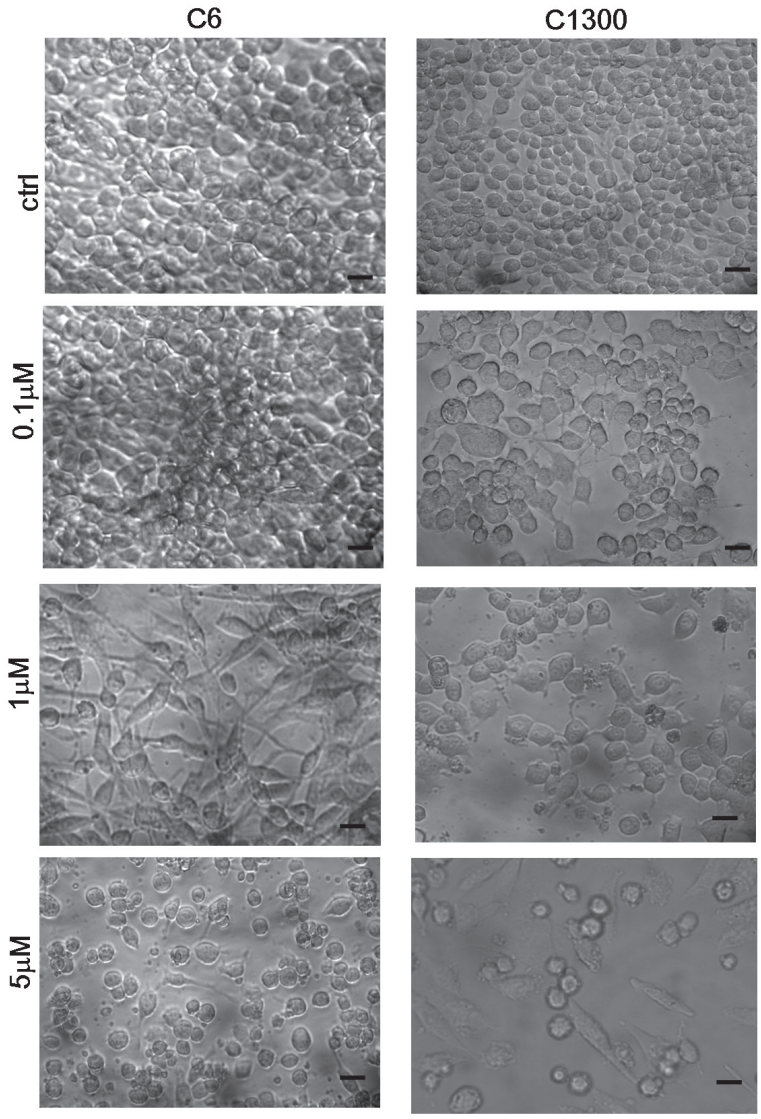

Fig. 1. - Contrast optic phase: 2ME exposure leads to reduction in number, globular/shrunken shape and retraction of cytoplasmic processes ranging from 0.1 μM and 1 μM in C1300 and C6 monolayers, respectively. Bar, 25 μm.

C6 cells had homogeneous blue nuclei (Hoechst 33342 blue-stained cells; Fig. 2). The decrease in cell number following 2ME-exposure was confirmed by the MTT test. Indeed, proliferation rate declined in a dose-dependent fashion in both monolayers (*%P* = 65 ± 7 at 0.1 μM, *%P* = 45 ± 9 at 1 μM and *%P* = 30 ± 5 at 5 μM in C1300 cells; *%P* = 85 ± 5 at 1 μM and *%P* = 58 ± 8 at 5 μM in C6 cells; Fig. 3); furthermore, IC_{50} was between 0.1 μM-1 μM and > 5 μM in C1300 and C6 cells, respectively. The concentrations tested in this work (0.1 μM, 1 μM and 5 μM) were in accordance with other experimental observations since 2ME did not exert significant effects at lower doses (data not shown), whereas at higher concentrations 2ME induced dramatic morpho-functional changes (data not shown).

In both cell cultures studied the bands obtained by western blot immunodetection were located at about 55 kDa. This finding was consistent with the molecular weight of tubulin, which is 55 kDa (Figs. 4, 5, 6). The Student's *t* test and one-way ANOVA performed on the acet-tub, tyr-tub and total α-tubulin expression showed a significant effect for 2ME. As demonstrated by the densitometric analyses of the bands, 2ME altered the expression of tyr-tub and total α-tubulin, whereas the acet-tub expression did not vary. In par-

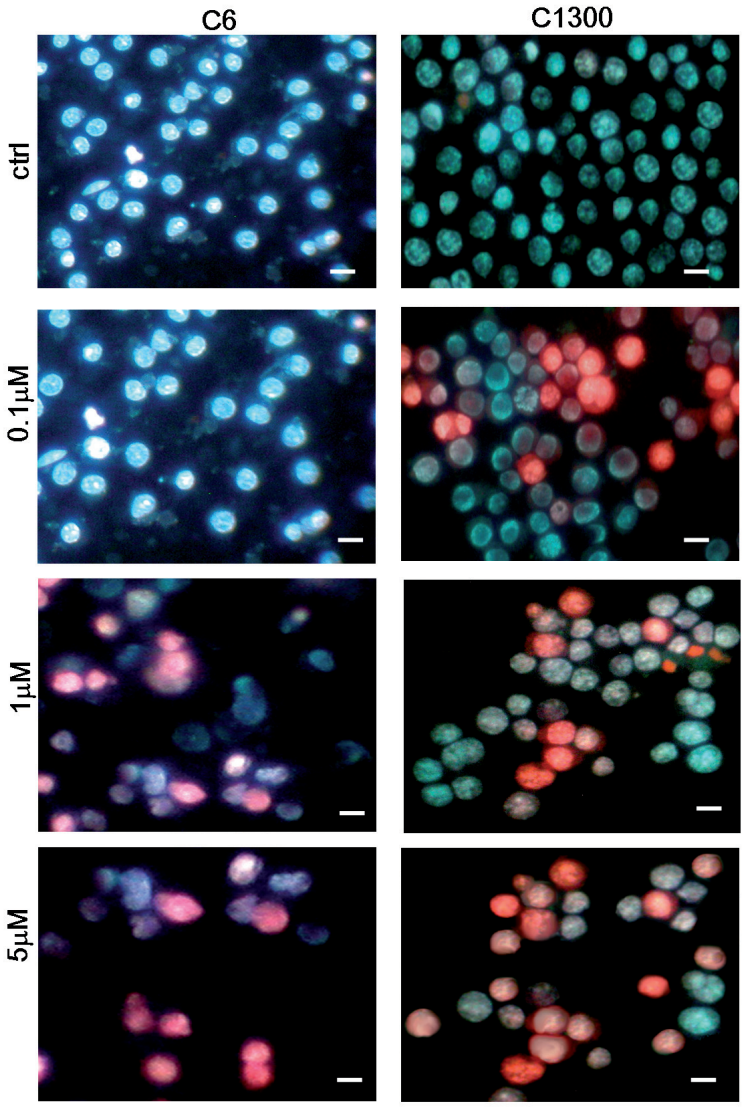

Fig. 2. - Viability assay: Hoechst H33342/PI staining. 2ME-exposed cells displayed a high number of dead cells (PI-stained red) and nuclear changes ranging from 0.1 μM in C1300 cells and from 0.1 μM in C6 monolayers. Bar, 25 μm.

ticular, the acet-tub values did not change following 2ME exposure at any concentrations tested in either cell culture (*%P* = 82 ± 7, *%P* = 87 ± 8, *%P* = 86 ± 9 in C1300 cells, *%P* = 89 ± 8, *%P* = 85 ± 6, *%P* = 88 ± 6 in C6 cells, at 0.1 μM, 1 μM and 5 μM, respectively; Fig. 4). By contrast, the tyr-tub expression declined in a dose-dependent manner in C1300 cells since the values were reduced at 0.1 μM (*%P* = 80 ± 6), at 1 μM (*%P* = 50 ± 7) and mostly at 5 μM (*%P* = 30 ± 5). In C6 cells the tyr-tub expression was significantly reduced only at the highest concentration used (*%P* = 65 ± 4), whereas a slight reduction in the expression of this isoform was observed at 0.1 μM and 1 μM (Fig. 5). Finally, 2ME did not modify the total α-tub expression at 0.1 μM in both cell cultures, whilst at 1 μM (*%P* = 42 ± 5 in C1300 cells, *%P* = 57 ± 9 in C6 cells, respectively) and 5 μM (*%P* = 51 ± 5 in C1300 cells, *%P* = 63 ± 4 in C6 cells, respectively) the values were similarly reduced in the two cell lines analyzed.

Discussion

Results demonstrate that 2ME is more effective in C1300 cells than in C6 cells. The observed morphological and functional changes induced by 2ME

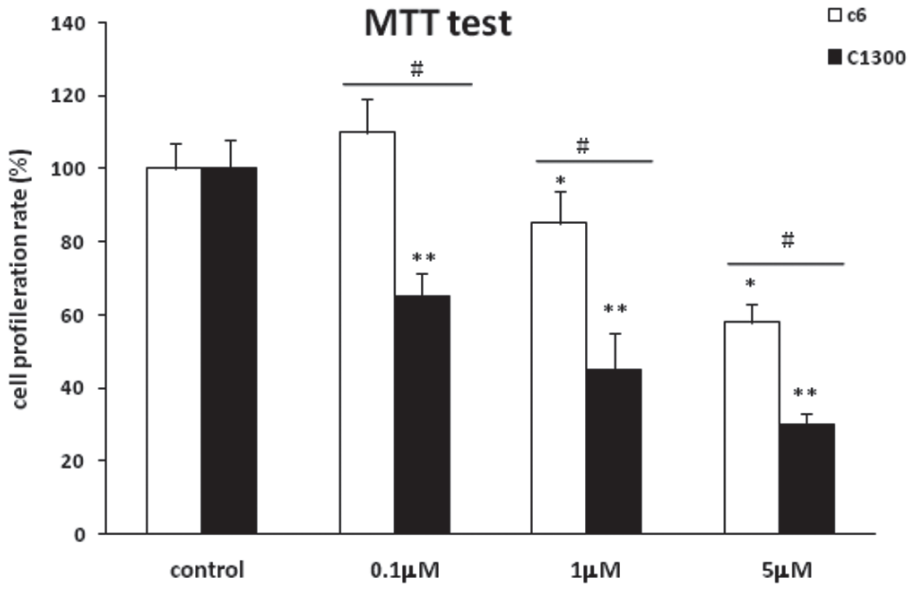

Fig. 3. - MTT test. Cell growth inhibition in C1300 and C6 cells 2ME-exposed. Cell proliferation is expressed as a percentage of the maximum value compared with the control cells (DMSO-treated). Dose-dependent decrease of values was higher in C1300 than in C6 cells. Each column refers to the mean ± S.E.M. $n = 6$. * $P < 0.05$ and ** $P < 0.01$ compared with corresponding DMSO-treated control cells. # $P < 0.05$ = between the two cell lines exposed to the same concentrations.

exposure are in accordance with other *in vitro* studies which were carried out on cells of both neuronal and glial origin (Nakagawa-Yagi et al., 1996; LaVallee et al., 2003; Braeuninger et al., 2005; Chamaon et al., 2005). In this study we show that 2ME effectiveness is in relationship with the specific cell phenotype since 2ME was found to induce more severe alterations in the C1300 cells than in the C6 cells. Indeed, morpho-functional changes detected by phase contrast optics and cell viability test were more remarkable in C1300 than in C6 cells; moreover, IC_{50} was about 1 µM and > 5 µM for C1300 and C6 cells, respectively. Additionally, in accordance with a previous study performed by Gokmen-Polar and co-workers (Gokmen-Polar et al., 2005), it was also found that 2ME alters the expression of specific tubulin isoforms more effectively in C1300 than in C6 cell cultures. The expression of tyr-tub and total α-tubulin decreased in a marked dose-dependent manner in C1300 cells, whereas such decrease was less significant in C6 cells at the same micromolar concentrations. The acet-tub expression was instead not significantly modified in either cell lines. The observed high reduction of the tyr-tub/total α-tubulin expression without concomitant significant modification of the acet-tub expression indicates an impairment of the microtubular network. The microtubular network is a dynamic cellular system able to assembly (polymerize), disassembly (depolymerize) and rearrange following intrinsic or extrinsic stimuli. These properties are due to the physiological features of the polymers, which derive from the biochemical properties of the αβ-heterodymer, the microtubule building block. Events like cellular division, cell adhesion, elongation of cytoplasmic processes, axonal and synaptic remodeling, neuronal differentiation and maturation need dynamic forms of microtubules (Meninger and Binet, 1989; Rochlin et al., 1996). By contrast, when the microtubular system is stable or less dynamic the cellular events above mentioned are significantly impaired. The severe reduction in the tyr-tub expression, as found in C1300 cells, supports an altered dynamic status of the microtubules (Gundersen et al., 1987; Rochlin et al., 1996) leading to changes in morphology, impairment of the microtubular system physiology, mitosis block and consequent cell death (Brueggemeier et al., 2001). The results of this study are in accordance with Gokmen-Polar and co-worker's findings, since a significant reduction

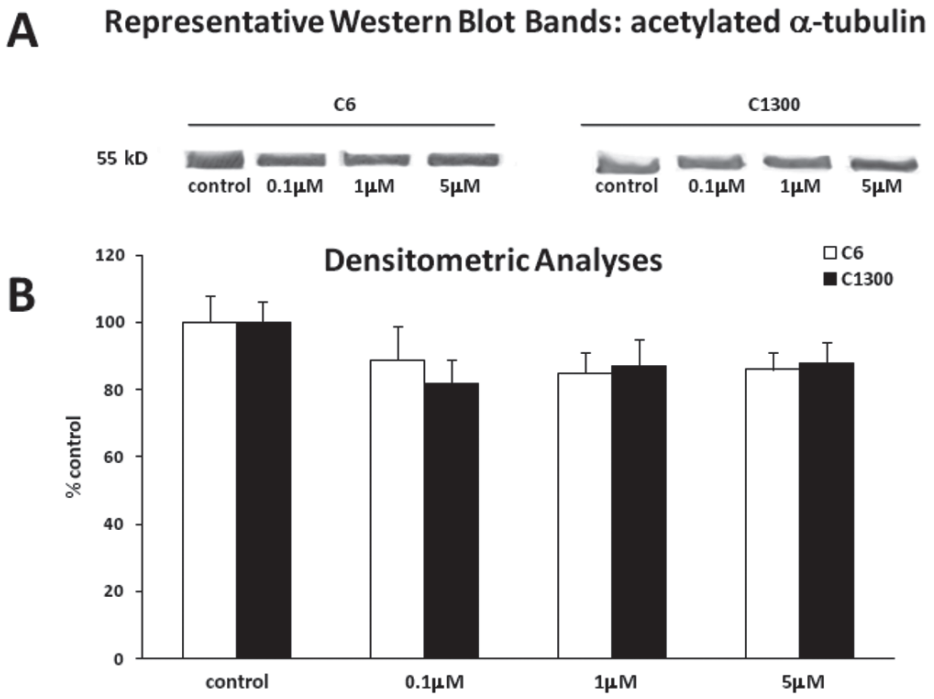

Fig. 4. - A: Representative western blots showing the acetylated α-tubulin immnunoreactivity in 2ME-exposed C1300 and C6 cells. B: Densitometric analysis obtained via western blot. 2ME did not significantly alter the expression of acet-tub at any concentrations tested both in C1300 and C6 cells. Each column refers to the mean ± S.E.M. *n* = 6.

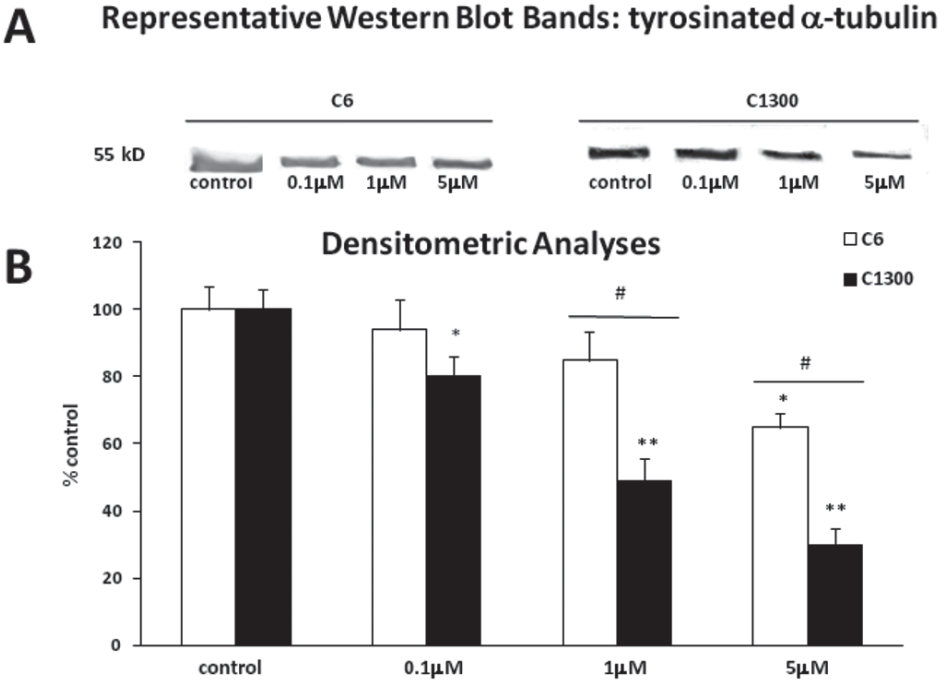

Fig. 5. - A: Representative western blots showing the tyrosinated α-tubulin immnunoreactivity in 2ME-exposed C6 and C1300 cells. B: Densitometric analyses obtained via western blot. A dose-dependent decrease of values was observed at 1 μM and 5 μM in both C1300 and C6 cells, but the decrease was significant ranging from 0.1 μM in C1300 cells. Each column refers to the mean SD ± of six single experiments. * $P = 0.05$, ** $P = 0.01$ compared with corresponding DMSO-treated control cells. # $P < 0.05$ = between the two cell lines exposed to the same concentrations.

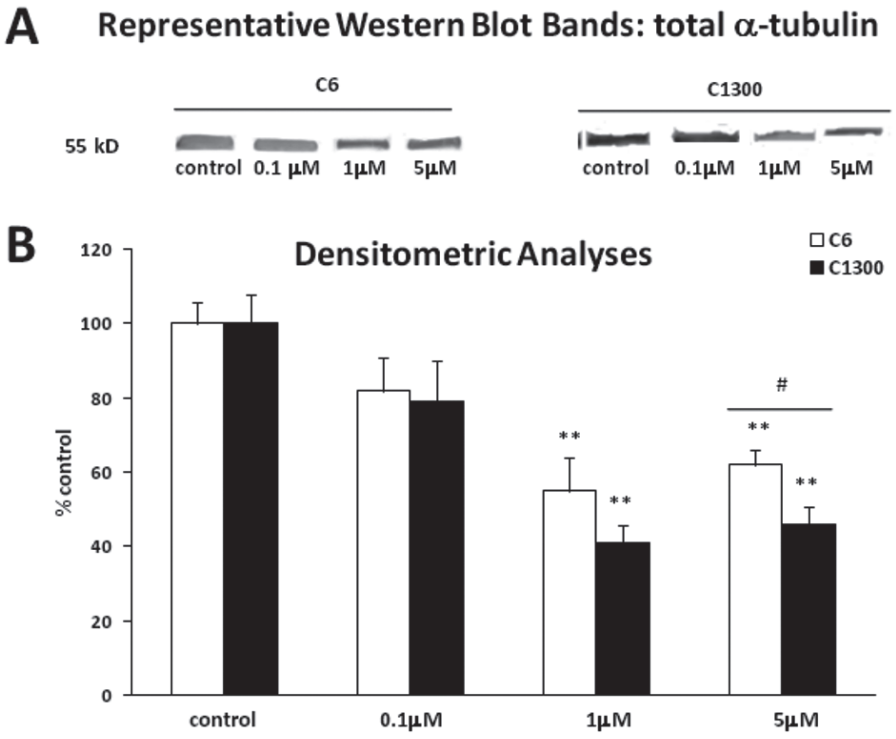

Fig. 6. - A: Representative western blots showing the total α-tubulin immnunoreactivity in C1300 and C6 cells 2ME-exposed. B: Densitometric analyses obtained via western blot. A dose-dependent decrease of values was higher in C1300 than in C6 cells at 1 μM and 5 μM. Each column refers to the mean ± S.E.M. $n = 6$. * $P = 0.05$, ** $P = 0.01$ compared with corresponding control cells DMSO-treated. # $P < 0.05$ = between the two cell lines exposed to the same concentrations.

of the tyr-tub expression has been also found in the MDA-MB-435, W435 and P435 cell cultures following 2ME-exposure (Gokmen-Polar et al., 2005).

In this preliminary study a compared analysis of the 2ME effectiveness in neural and glial cells was presented for the first time. On the basis of these results and further data not yet published, we reasonably conclude that 2ME exposure induces morpho-functional changes and impairs the microtubular system physiology more noticeably in neural cells than in glial cells. This finding is confirmed by further experimental data (not yet published), since neuronal cells were found to be more sensitive than glial cells, not only in neoplastic high proliferating cells but also in normal slow-proliferating C1300/C6 cell cultures as well (data not shown). However, this assumption partially disagrees with other published reports. Given the fact that glial cells have a much higher proliferation rate than neuronal cells, 2ME should be more effective in glial than in neural cells (Seegers et al., 1997; Brueggemeier et al., 2001). Therefore, other molecular mechanisms should be taken into account in order to explain the specific 2ME effectiveness on C1300 and C6 cells.

Firstly, it can be hypothesized that, independently from the peculiar proliferating activity of the cell phenotype investigated, the sensitiveness of neural cells could be linked to the high 2ME affinity with α and β tubulin isoforms, the patterns of which are manifested to be more noticeably in neural cells than in glial cells (Moura Neto et al., 1983). As a consequence, the perturbation of tubulin dynamics, resulting in an impairment of the microtubular system physiology, is more intense in C1300 cells than in C6 cells. This finding is in accordance with the severe decrease of the tyr-tub not accompanied by variation of the acet-tub expression found by some Authors in other 2ME-exposed cell types (Piperno et al., 1987; Rochlin et al., 1996).

Alternatively, the extreme toxicity of 2ME in C1300 cells may be due to an impairment of the general metabolic patterns. A perturbation of protein syn-

thesis has been demonstrated by Nagakawa-Yagi (Nakagawa-Yagi et al., 1996) in 2ME-exposed neuroblastoma cells even if the intrinsic mechanism by which 2ME exerts its action has not been yet discovered or suggested. We hypothesize that such 2ME-induced metabolic perturbation may result in excitoxicity phenomena. Neuronal and glial excitoxicity comes from the specific neuronal and glial physiological features. Neurons, especially activated neurons, have a higher energy requirement than glial cells. Since neurons only produce glutamate needing higher amounts of glucose and lactate than glial cells, if glucose and lactate are in short supply, neuronal cells are more likely to starve than glial cells. Thus, any metabolic failure determines a greater cellular damage in neuronal than in glial cells. 2ME-induced metabolic perturbations may result in an energy depletion which is higher in C1300 than in C6 cells followed by an immediate breakdown of cellular functions and consequent excitotoxicity phenomena. In conclusion, in this *in vitro* study, we observed that C1300 and C6 cells were both sensitive to 2ME toxicity, although neural cells appeared to be more vulnerable than glial cells. We also postulate that the significant 2ME-induced morphological and functional changes, could be specifically caused by an impairment of the microtubular system. An interaction/incorporation of 2ME into α-tubulin isoforms, resulting in marked alterations of the tyr-tub expression, could be one among other mechanisms by which 2ME exerts its activity. A perturbation of the metabolic patterns with consequent excitotoxicity phenomena could also explain the different sensitiveness of neural and glial cells to 2ME. Reports regarding the *in vivo* effects of 2ME on neural and glial cells are quite scarce. However, the observed capability of 2ME to induce significant loss of neurons in the rat ippocampus hilus (Picazo et al., 2003), or cell growth inhibition in some variety of orthopic glial tumor models in rats and mice are in accordance with these results (Kang et al., 2006; Plum et al., 2006). Moreover, as also postulated in this work, these Authors conclude that the impairment of microtubules system in actively diving cells, is most likely to be the main mechanism by which 2ME inhibits cell growth in the tumor models studied. Other specific mechanisms of 2ME *in vivo* action in nerve and glial cells have not been studied and need to be further investigated.

For this reason, though the *in vitro* system proposed is not without limitations, it could represent a suitable model for further studies aimed at investigating the ways in which the ability of 2ME to impair the microtubular system, perturb cellular methabolism and exert its cytotoxic effects are linked.

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