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Curcumin potentiates the antitumor activity of Paclitaxel in rat glioma C6 cells

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ABSTRACT

Background: Glioma is the most common primary cancer in central nervous system, especially in brain. Paclitaxel (PTX) is a microtubule stabilizing agent with anticancer potential, but its clinical application to brain tumours is limited by drug resistance, side effects, and lower brain penetration.
Purpose: Herein we explored the *in vitro* effects, in glioma C6 cells, of the combination of PTX with curcumin, a natural compound with chemotherapeutic activity, in order to improve cytotoxic effects and overcome PTX limitations.
Results: Our data confirmed PTX antiproliferative activity, that was improved by curcumin. These effects were confirmed by clonogenic assay and G0/G1 cell cycle arrest. PTX significantly promoted generation of intracellular reactive species (RS), while curcumin did not affect RS production; the combination of the two drugs resulted in a slight but significant increase in RS levels. Furthermore, we found a constitutive activation of NF-κB in C6 cell line that was inhibited by PTX and curcumin. Interestingly, combination of the drugs totally inhibited NF-κB nuclear translocation and reduced IκB phosphorylation. Our results also supported the involvement of p53-p21 axis in the anticancer effects of curcumin and PTX. The combination of the two drugs further increased p53 and p21 levels enhancing the antiproliferative effects. Furthermore, PTX plus curcumin most impressively activated caspase-3, effector of apoptosis pathways, and reduced the expression of the anti-apoptotic protein Bcl-2.
Conclusion: In conclusion, our findings demonstrated that combination of PTX and curcumin exerts a potentiated anti-glioma efficacy *in vitro* that may help in reducing dosage and/or minimizing side effects of cytotoxic therapy.

Keywords: Curcumin; Paclitaxel; Antiproliferative; Glioma; NF-κB; p53.

Abbreviations: Cur, curcumin; RS, reactive species; PTX, paclitaxel

Introduction

Despite the low percentage of cases, cerebral glioma possesses high mortality and morbidity rate. Especially, glioblastoma is the most aggressive and malignant brain cancer with rapid development. Furthermore, glioma cells possess enhanced resistance to radiotherapy- and chemotherapy-induced death (Omuro and DeAngelis, 2013).

Paclitaxel (PTX), a microtubule stabilizing agent with highly potent anticancer potential, is recommended as a first-line strategy chemotherapeutic agent against many kinds of cancers. However, the clinic efficacy of PTX in brain tumor is limited by the drug resistance, besides serious several side effects and limited bioavailability and cancer penetration (Marupudi *et al.*, 2007; de Weger *et al.*, 2014).

Due to the limited therapeutic efficacy of monotherapy, current clinical practice for treating cancer includes the co-administration of more molecular targeted drugs acting at multiple molecular levels on a single cellular pathway or on parallel pathways, as well as addressing the same target through different mechanisms of actions, to maximize efficacy, minimize toxicity and overcome cell resistance to chemotherapy. In this scenario, curcumin, a yellow pigment from the spice turmeric (*Curcuma longa*), has been reported for its potential chemopreventive and chemotherapeutic activity through influence on cell cycle arrest, differentiation, and apoptosis in different kinds of cancer (Surh and Chun, 2007). ~~Although curcumin targets many transcription factors (ATF3, AP-1, STAT-3), protein kinases, enzymes, growth factors, inflammatory mediators, and anti-apoptotic proteins (Luthra and Lal, 2016),~~ Among the molecular targets of curcumin, there has been considerable interest in its ability to inhibit NF-κB pathway activity (Dhandapani *et al.*, 2007). This is relevant since there is large evidence that the transcription factor NF-κB plays a main role in the control of oncogenesis, tumor progression and chemotherapy resistance of diverse types of malignances (Nakanishi and Toi, 2005)~~. NF-κB is a homo or heterodimer present as an inactive cytoplasmic complex by interaction with inhibitory proteins of the IκB family. Upon stimulation, IκBs are phosphorylated by IKK (IκB kinase) proteins thus releasing the active NF-κB, which translocates into nucleus so activating genes involved in inflammation (IL1-beta, IL-6, COX2, and TNF), apoptosis resistance (bcl-xL, cIAP1/2), cell invasion (ICAM-1, MMPs), angiogenesis (VEGF), proliferation (cyclins) and metastasis (Nakanishi and Toi, 2005)~~. Aberrant NF-κB activity has been reported in cancer cells and analysis of brain tumor biopsies ~~showing that NF-κB and its target genes are overexpressed in glioblastoma and astrocytoma tumors compared to normal brain tissues (Raychaudhuri *et al.*, 2007). Besides, constitutive NF-κB up-regulation~~ and has been found in chemotherapy-resistant cell lines, which correlates with therapy failure (Orlowski and Baldwin, 2002). In this context, studies have suggested that compounds with NF-κB inhibitory properties are

potential anticancer agents (Dhandapani *et al.*, 2007) efficient also in glioblastomas (Zanotto-Filho *et al.*, 2011). Zanotto-Filho and coworkers (Zanotto-Filho *et al.*, 2012) demonstrated that in C6 and U138MG glioblastoma cells curcumin decreased the constitutive activation of PI3K/Akt and NF-κB survival pathways and down-regulated the antiapoptotic NF-κB-regulated protein bcl-xl. Thus, curcumin may be a potential adjuvant by decreasing the antiapoptotic threshold, leading to sensitization of glioblastoma cells to apoptotic stimuli triggered by chemotherapic drugs. Taken into account this background, in view of a possible employment of the two drugs curcumin and PTX combined together to overcome PTX limitations, the present study was undertaken in order to investigate, through in vitro experiments on rat glioma C6 cells, the capability of curcumin to enhance the sensitivity of glioma cells to by modulating the activity of specific cell signalling pathways.

Materials and methods

Reagents

PTX (≥ 98% purity) and curcumin (≥ 90% purity) were supplied from Cayman Chemical (Ann Arbor, MI, USA). All other reagents, unless otherwise specified, were purchased from Sigma Aldrich (Milan, Italy).

Cell cultures and treatments

The rat C6 glioma cell line was obtained from the American Type Culture Collection (ATCC®) (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) plus penicillin and streptomycin.
C6 glioma cell is a rat cell line of glioblastoma multiforme (GBM) that has the ability to form tumors in vivo and share several malignant characteristics with the human GBM (Barth *et al.*, 2009). Moreover, C6 glioma model is surely one of the best and widely characterized about the genic and proteic profile, being successfully used to investigate the mechanism of tumor growth, angiogenesis and invasion, to characterize the molecular mechanisms involved in the effect of antineoplastic drugs (such as taxol) and to design new anticancer therapies (Chao *et al.*, 2015).For all the experiments, curcumin and PTX were always freshly dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture medium during the different treatments was always maintained at 0.05% (v/v). Subconfluent cells were treated for 48 h with PTX (12.5-100 nM), curcumin (5-10 μM), or with combinations of curcumin and PTX. Cultures treated with the vehicle alone (0.05% DMSO) were used as controls (CTR).

MTT assay

Cells were plated into 24-well plates at a density of 8 x 10^4 cells/well in 500 µl medium, and were treated with PTX (12.5-100 nM), curcumin (5-10 µM), or with combinations of curcumin (5-10 µM) and PTX (12.5-25 nM) for 48 h. The 48 h exposure time was selected because in a preliminary set of experiments 24 h exposure to curcumin or PTX alone induced only a moderate cytotoxic effect (data not shown). After the exposure time, the MTT assay was performed according to Dang and coworkers (Dang *et al.*, 2015).

Results are reported as percentage of viable cells calculated from the number of viable cells in treated samples vs control (cultures treated with the vehicle alone, 0.05% DMSO). The 50% and 30% lethal concentrations (LC_{50} and LC_{30}), defined as the concentration needed to kill 50% (or 30%) of the treated cells in comparison with untreated control cells, and 90% confidence limits (C.L.) were calculated using the Litchfield and Wilcoxon test.

Isobologram analysis has been used as a method for identifying the combined effect of multiple drugs in terms of the additive, synergistic, or antagonistic effect. Briefly, LC_{50} and LC_{30} values of curcumin and PTX were plotted on the x- and y-axes, respectively, to form two straight lines. The data point of the isobologram corresponds to the actual LC_{50} (or LC_{30}) value of combining PTX with curcumin. If a data point is on or near the line, this represents an additive effect, a data point below the line indicates synergism, and a data point above the line represents antagonism.

Clonogenic assay

Clonogenic assay was accomplished on C6 cells by plating 1 x 10^5 cells/well in 6-wells plates. After 24 h, cells were treated for 48 h with different doses of curcumin (5 – 10 µM) and/or PTX (5 – 15 nM); control cells were treated with the vehicle alone (DMSO, 0.05%). Clonogenic survival test was performed as described by Anwar and coworkers (Anwar *et al.*, 2016). The colony-forming efficiency was calculated as a percentage with respect to control cells.

Intracellular reactive species measurement by DCFH-DA assay

Intracellular reactive species (RS) were measured using the oxidation-sensitive fluorescent probe, dichloro-dihydro-fluorescein diacetate (DCFH-DA) as described by Fratantonio and coworkers (Fratantonio *et al.*, 2015). RS levels were measured as DCFH-DA relative fluorescence intensity and expressed as fold change vs control.

Western blot analysis

For immunoblot analyses, 40 µg of protein of cellular and nuclear lysates were subjected to SDS-PAGE according to Fratantonio and coworkers (Fratantonio *et al.*, 2015). Membranes were then probed with specific primary antibodies (from Santa Cruz Biotechnology): caspase-3 rabbit mAb (1:1.500); Bcl-2 rabbit mAb (1:1.000); phospho-p53 rabbit pAb (1:1.000); $p21^{Waf1/Cip1}$ rabbit mAb (1:1.000); phospho-IκB-α rabbit mAb (1:1000); NF-κB (p65) rabbit mAb (1:1000); β-actin rabbit mAb (1:6.000); Lamin B mouse mAb (1:600), followed by peroxidase-conjugated secondary antibody, and visualized with an ECL plus detection system (Amersham Biosciences). Densitometry was performed using ImageJ software (https://imagej.nih.gov/ij/).

Cell cycle analysis

Cell cycle distribution was performed according to Allegra and coworkers (Allegra *et al.*, 2018).

Statistical analysis

All the experiments were performed in triplicate and repeated three times. Results are expressed as mean ± SD from three experiments and statistically analyzed by a one-way or a two-way ANOVA test, followed by Tukey's HSD, using the statistical software ezANOVA (http://www.sph.sc.edu/comd/rorden/ezanova/home.html). Differences in groups and treatments were considered significant for $P < 0.05$.

Results

Cytotoxic and antiproliferative effect of PTX and curcumin

To evaluate the cytotoxic effect of PTX and curcumin, C6 glioma cells were treated with the two compounds alone or in combination. A 48 h treatment with PTX alone induces significant and dose dependent cell mortality, although showing an initial sharp decline in cell survival until 25 nM, whereas above this dose there is only a modest increase in cytotoxicity (Liebmann *et al.*, 1993). Curcumin alone induced only a slight (although statistically significant) decrease in cell viability at both the tested doses (5 and 10 µM) but significantly improved the cytotoxic effect of PTX 12.5 and 25 nM (Fig. 1). ~~These doses of PTX (12.5-25 nM) and curcumin (5 and 10 µM) are able, if used in combination, to give an (at least) additive effect as calculated by using the Chou-Talalay combination index method (Chou, 2010).~~ The doses of PTX (25 nM) and curcumin (5-10 µM) used throughout the study were chosen since they are able, if used in combination, to give a synergistic cytotoxic effect as calculated by using the Chou-Talalay combination index (C.I.) method (Chou, 2010), with C.I. values of 0.52 and 0.67 for 25 nM PTX in combination with 5 and 10 µM

curcumin, respectively. The combination of PTX with curcumin 5 or 10 μM, in fact, lowered PTX LC_{50} from 68 nM to 46 and 24 nM, respectively (Fig. 1; see ~~Fig.1S~~Supplementary material for the complete dose-response curves used for LC_{50} determination). The antiproliferative effect of PTX and curcumin on C6 glioma cells was also evaluated by means of the clonogenic assay. This test, also known as colony forming assay, is based on the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony. For the clonogenic assay, C6 glioma cells have been treated for 48 h with PTX (5-15 nM) and/or curcumin (5-10 μM), and the formed cell colonies were counted 7 days after seeding. Due to the sensitivity of C6 cells to PTX in this kind of test, lower doses of PTX (5-15 nM) able to produce only weak/moderate decrease in cell viability assay were tested. Both PTX and curcumin dose-dependently inhibited the capacity of C6 cells to form colonies (Fig. 2). Interestingly, combination of 5 nM PTX with 5 μM curcumin was sufficient to significantly reduce ~~significantly~~ clonogenicity in C6 cells with respect to control.

Effects of PTX and/or curcumin on cell cycle arrest

The results showed in Fig. 3 depict a representative profile of cell cycle distribution in C6 cells following different treatments. In the control group, cells without drug exposure presented 78.9% and 19.2% populations in G0/G1 and S phase, respectively. Treatment with PTX caused a slight accumulation of the G0/G1 fraction (up to 87.7%; $p < 0.05$ vs CTR) and a reduction of S phase (up to 8.7%; $p < 0.05$ vs CTR). Curcumin treatment did not affect cell cycle at all the concentrations used. Interestingly, PTX-curcumin combinations further increased the G0/G1 fraction and reduced S phase up to 90.7% and 2.1% respectively ($p < 0.05$ vs CTR; $p < 0.05$ vs PTX treatment), indicating that this drug association is able to halt cells in G0/G1 phase and promotes additional S phase reduction.

Curcumin potentiates PTX-induced RS accumulation in C6 glioma cells

Studies have shown that, when compared to normal cells, cancer cells can be rendered susceptible to reactive oxygen species (ROS)-mediated cytotoxicity, because they are intrinsically under oxidative stress due to increased steady-state levels of $O_2^{\bullet -}$ and H_2O_2 from mitochondrial metabolism.

So, we measured intracellular RS levels by the DCFH-DA assay under our experimental conditions. While PTX 25 nM increased intracellular RS accumulation by almost 2-fold, RS levels were not significantly increased by curcumin 5-10 μM, when compared ~~with~~ to the control (Fig. 4). However, the combination of PTX and curcumin increased RS accumulation in comparison with

PTX alone. Thus, these results suggest that the synergistic antiproliferative effect of curcumin and PTX may be associated with a deeper intracellular RS accumulation.

PTX and curcumin inhibit NF-κB signaling pathway in C6 glioma cells

The transcription factor NF-κB is constitutively over-expressed in malignant glioma cells, an aberration associated with enhanced growth, cell cycle progression, and inducible chemoresistance (Dhandapani *et al.*, 2007; Raychaudhuri *et al.*, 2007), thus it may influence tumor progression and represents a potential target for glioblastoma therapy. In our experimental conditions, PTX 25 nM and curcumin 5 or 10 μM reduced phosphorylation of NF-κB inhibitor IκB and then induced a down-regulation of NF-κB nuclear accumulation (Fig. 5). Down-regulation of this signaling pathway was slightly stronger in cells treated with the PTX-curcumin combinations, especially for PTX and Cur 10 μM, then in those treated with one of the two compounds alone.

PTX and curcumin modulate the p53-p21 axis activity in C6 glioma cells

NF-κB exerts its function mainly as an antagonist to p53 transactivation, a key tumor suppressor protein. The unlimited replication potential of cancer cells can result from the inactivation of p53 since it regulates molecules involved in apoptotic pathways and cell cycle regulation. The cell cycle-related protein $p21^{Waf1/Cip1}$, a CDK2 and CDK1 inhibitory protein transcriptionally regulated by p53, causes G1 cell cycle arrest (Waldman *et al.*, 1995). PTX 25 nM and curcumin 5 or 10 μM induced the p53-p21 axis activity when compared to control cells (Fig. 6). Moreover, the two tested PTX-curcumin combinations further upregulated both proteins, showing a deeper effect on cell cycle regulation than that of the two compounds when used alone.

PTX and curcumin induce apoptosis in C6 glioma cells

Caspase-3, a cytosolic protein normally present as an inactive precursor (pro-caspase-3), is proteolytically cleaved into a low molecular weight (17 kDa) active enzyme when cells undergo apoptosis (Shalini *et al.*, 2015). To determine the proapoptotic effect of PTX and curcumin we evaluated cleaved caspase-3 in C6 treated cells. Both PTX and curcumin induced caspase-3 activation at the tested doses (Fig. 7). Moreover, combination with curcumin significantly enhanced PTX proapoptotic effect, with increased caspase-3 levels compared to PTX or curcumin alone. Moreover, we evaluated the modulation of the anti-apoptotic Bcl-2 proteins, up-regulation of which has been implicated in the resistance of many cancer cells (Skommer *et al.*, 2010). Interestingly, the proapoptotic effect of curcumin, PTX, and, more efficiently, of the two drugs used in combination,

is evidenced by the significant down-regulation of the pro-survival/antiapoptotic protein Bcl-2 (Fig. 7).

Discussion

Glioma is the most common primary cancer in central nervous system, especially in brain. At present, there are not many choices for the chemotherapy of patients with malignant glioma and the first-line strategies of brain glioma therapy are still mainly alkylating agents concomitant with or after radiotherapy. PTX, a microtubule stabilizing agent with highly potent anticancer potential, is recommended as a first-line chemotherapic strategy against many kinds of cancers, but its clinic application to brain tumours is limited by drug resistance, as well as side effects and lower brain penetration (de Weger *et al.*, 2014). However, the fundamental challenge to the discovery of new, efficient microtubule-targeting drugs is represented by the existence of drug resistance mechanisms (Katsetos *et al.*, 2015).

Combination therapy is a common way of clinical oncology chemotherapy able to improve treatment response, overcome drug resistance or minimize adverse events. Ramachandran et al. have shown that curcumin could be used to ameliorate the therapeutic potential of temozolomide or etoposide in brain tumor cells (Ramachandran *et al.*, 2012). Because curcumin and PTX produce their antitumor effect by different mechanisms, we were interested in studying their combined effect on glioma cells. Our data confirmed the strong inhibition rate of PTX on glioma C6 cells (LC_{50} 68 nM) (Fig. 1). Interestingly, combination with curcumin increased cytotoxic effects of PTX in a dose-dependent way, so that a significant decrease of LC_{50} values was observed. These effects were also confirmed with the clonogenic assay (Fig. 2); in fact, PTX and curcumin combinations reduced C6 clonogenic survival more than the single drug so supporting an improved anticancer effect when the drugs are used together.

We also demonstrated G0/G1 cell cycle arrest for PTX and a reduction of S phase in glioma C6 cells while curcumin did not affect cell cycle distribution (Fig. 3). Interestingly, PTX-curcumin combinations further increased the fraction of cells in G0/G1 while markedly reduced S phase to 2.1%.

Increasing evidence demonstrates that the accumulation of ROS is correlated with the cytotoxic response induced by several chemotherapy agents. Importantly, chemotherapeutic drugs are selectively toxic to cancer cells since cancer cells appear to produce ROS at a greater rate than normal cells (Schumacker, 2006). It is not completely understood whether the PTX-induced modulation of ROS is related to its toxic effect in cancer cells; however Woo et al. reported that when glioma U373MG cells were treated with PTX in the presence or absence of N-acetyl-L-

cysteine (NAC), a widely used thiol-containing antioxidant, PTX-induced apoptosis was not affected so suggesting that ROS might be not directly involved in PTX-induced cell death (Woo *et al.*, 2008). In our experimental conditions, PTX significantly promoted the generation of RS in glioma C6 cells while curcumin did not affect RS production (Fig. 4). Interestingly, drugs combination resulted in a slight but significant increase in RS levels so that raising the level of RS above a toxic threshold can selectively kill cancer cells.

Aberrant activation of NF-κB has been reported in glioblastoma biopsies; in fact, in high-grade astrocytoma and glioblastoma, a positive correlation between NF-κB activation and glioma progression was observed (Raychaudhuri *et al.*, 2007) so that this pathway may become an important therapeutic target for these tumors. We found a constitutive activation of NF-κB in glioma C6 cell line (Fig. 5). PTX and curcumin showed inhibitory activity on p65 nuclear translocation and reduced phosphorylation of cytoplasmic NF-κB inhibitor p-IκB. These data are consistent with that reported in literature describing inhibitory activity of PTX and curcumin on NF-κB pathway (Dhandapani *et al.*, 2007; Karmakar *et al.*, 2008). However, our results interestingly demonstrated that combination of two drugs totally inhibited NF-κB pathway as observed by the lack of p65 nuclear levels. Similarly, we have previously observed that in multiple myeloma U266 cells combined treatment with carfilzomib and curcumin was significantly more effective in reducing NF-κB nuclear accumulation than cell exposure to carfilzomib or curcumin alone (Allegra *et al.*, 2018).

In agreement with our findings, other Authors have reported that curcuma is able to improve PTX-induced apoptosis through down-regulation of NF-κB pathway in hepatocellular carcinoma cells (Zhou *et al.*, 2015), human cervical cancer cells (Dang *et al.*, 2015) and human breast cancer cells (Zhan *et al.*, 2014).

Many studies reported the ability of NF-κB to suppress the p53-p21 axis thus inhibiting the induction of apoptosis (Gasparini *et al.*, 2014). Activation of the tumor suppressor p53 generally modulates various growth inhibitory or apoptotic genes and its activities play important roles in preventing cancer development. In particular, induction of p21, a cyclin-dependent kinase inhibitor, by p53 is able to produce cell cycle arrest. However, p21 expression has been shown to be regulated largely at transcriptional level by both p53-dependent and -independent mechanisms. The p21 promoter contains two conserved p53-binding sites, and at least one of them is required for p53 responsiveness after DNA damage. Our data demonstrated that PTX and curcumin were able to induce p53 but combination of the two drugs increased more significantly its levels (Fig. 6). Furthermore, we observed the same activation trend for p21 with an improved effect for the

association. These results then support the involvement of a p53-dependent p21 activation in the antiproliferative and proapoptotic effects of curcumin and PTX.

Given the importance of apoptosis in inhibiting clonogenicity survival of tumor cells *in vitro*, and since curcumin is able to inhibit growth of human glioblastoma A172, KNS60 and U251MG(KO) cell lines through a significant increase in caspase 3/7 activity, overexpression of BAX and Bcl-2 downregulation (Klinger and Mittal, 2016), we investigated also the involvement of these mechanisms in the glioma C6 cell death induced by PTX and curcumin. The combination therapy with curcumin and PTX most impressively activated caspase-3 (Fig. 7), the effector of extrinsic and intrinsic apoptosis pathway, in C6 cells. It also reduced the expression of the anti-apoptotic protein Bcl-2 (Fig. 7) more highly than the single drugs so as to favour apoptotic death via intrinsic pathway. George et al. have demonstrated that Bcl-2 siRNA significantly augmented taxol mediated apoptosis in different human glioblastoma cells through induction of calpain and caspase proteolytic activities (George *et al.*, 2009). Thus, our data contribute to support the hypothesis that combination of taxol and Bcl-2 inhibitors offers a novel therapeutic strategy for controlling the malignant growth of human glioblastoma cells. Similar effects were also demonstrated in human brain cancer HBTSC, LN18 and U138-MG cells where curcumin and PTX act synergistically with much greater activity than that seen with each individual agent in increasing Bax:Bcl-2 ratio, cytochrome C release and apoptosis (Hossain *et al.*, 2012).

All these mechanisms may contribute to PTX- and curcumin-induced cellular apoptosis, however, other pathways such as cell cycle regulation might also play a role, since PTX exposure essentially elicits G2/M accumulation of glioma cells (Janardhanan *et al.*, 2009; Cheng *et al.*, 2016). In conclusion, the major finding of our research presented is that curcumin plus PTX exert a potentiated anti-glioma efficacy in vitro, in which the combination resulted in the significant enhancements of glioma cell proliferation inhibition and apoptosis induction in vitro. The modulation of aberrant NF-κB activity and restoration of p53-p21 axis function represent the main molecular mechanisms involved in the observed effects. The data on glioma C6 cells in our research may help in reducing dosage and/or in minimizing side effects of cytotoxic therapy.

Conflict of interest

None

Legends

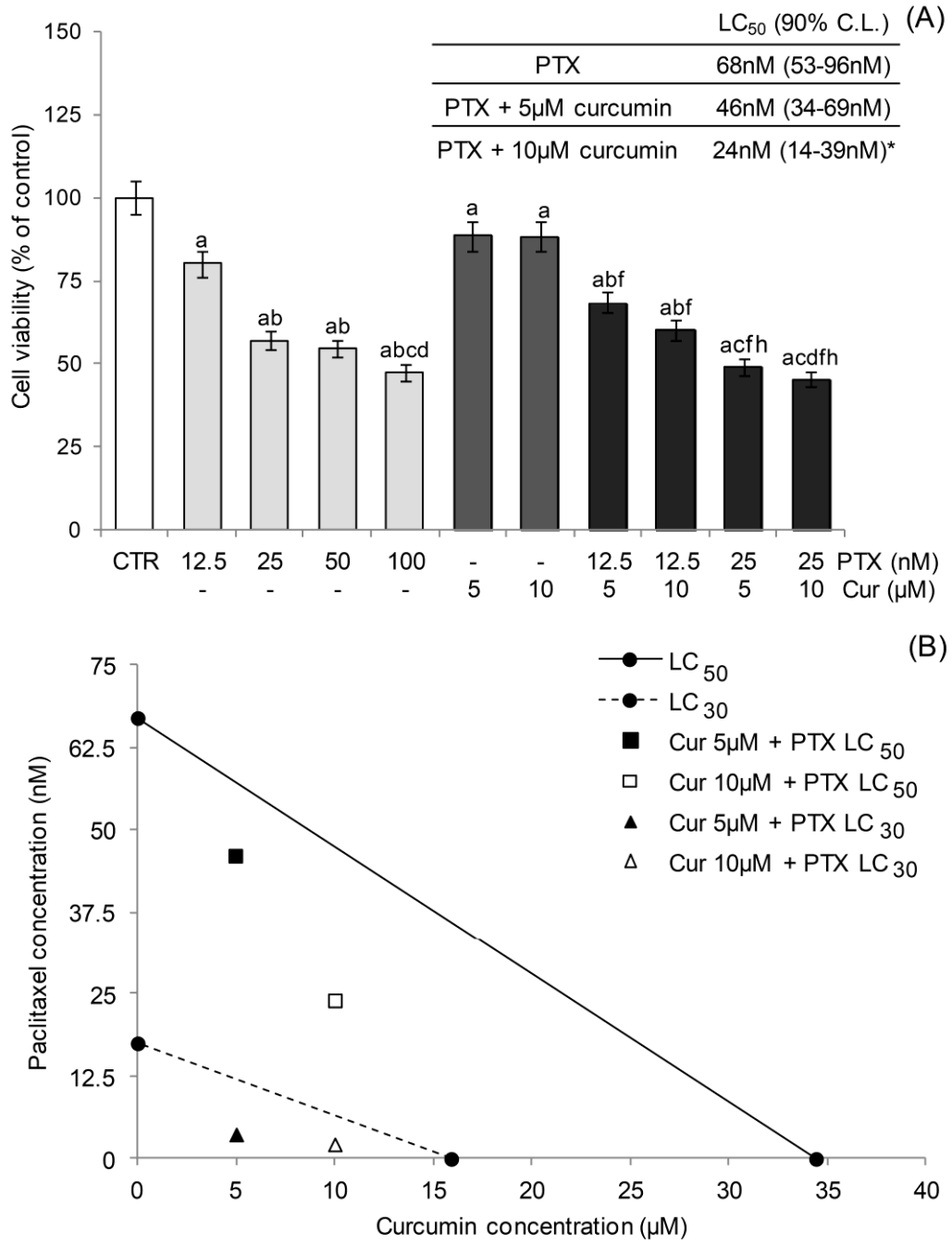

Fig. 1. MTT assay – C6 glioma cells were treated for 48 h with PTX (range 12.5-100 nM), Cur (5-10 μM) or with a combination of Cur (5-10 μM) and PTX (12.5-25 nM). (A) Cell viability data represent percentage of viable cells in treated samples vs CTR. Data are expressed as concentration producing 50% of cell death (LC_{50}) and 90% confidence limits (C.L.). (B) Isobologram analysis of the cytotoxic effects of PTX in combination with Cur. $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs 12.5 nM PTX; $^{c}p < 0.05$ vs 25 nM PTX; $^{d}p < 0.05$ vs 50 nM PTX; $^{f}p < 0.05$ vs Cur (5 and 10 μM); $^{h}p < 0.05$ 12.5 nM PTX plus Cur (5 and 10 μM); $^{*}p < 0.05$ vs PTX alone.

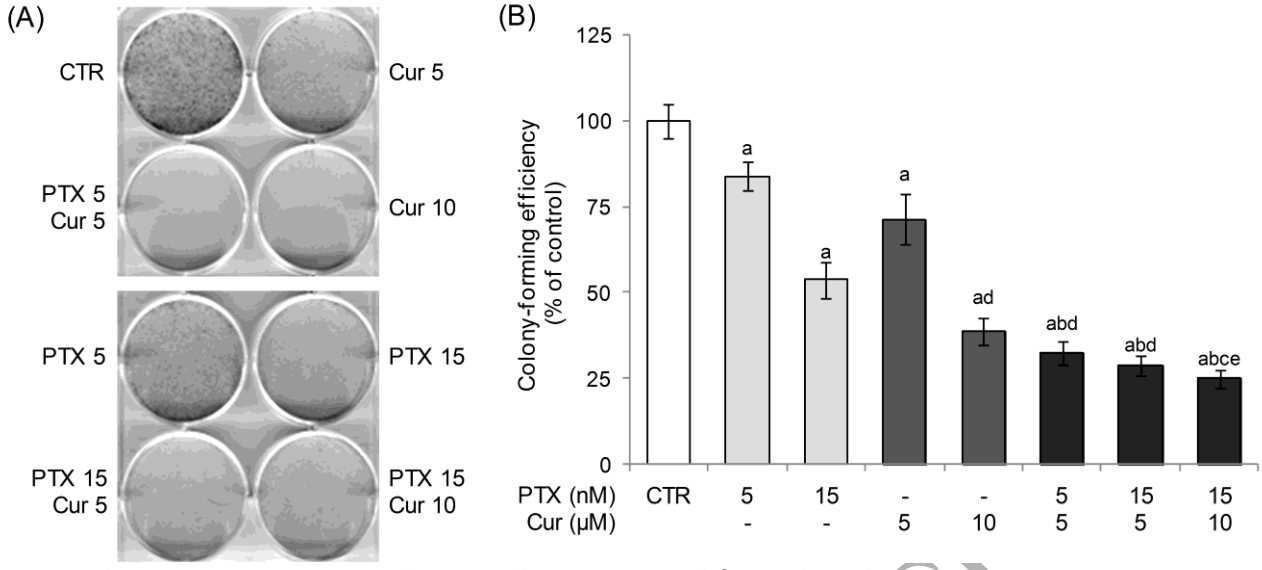

Fig. 2. Clonogenic assay - C6 glioma cells were treated for 48 h with PTX (5-15 nM), Cur (5-10 μM) or with a combination of Cur (5-10 μM) and PTX (5-15 nM). (A) Representative images of three independent experiments. (B) Data are expressed as colony-forming efficiency calculated as percentage with respect to CTR. Each point represents the mean ± SD of three experiments. $^{a}p < 0.05$ vs CTR; $^{b}p < 0.05$ vs 5 nM PTX; $^{d}p < 0.05$ vs 5 μM Cur; $^{e}p < 0.05$ vs 10 μM Cur.

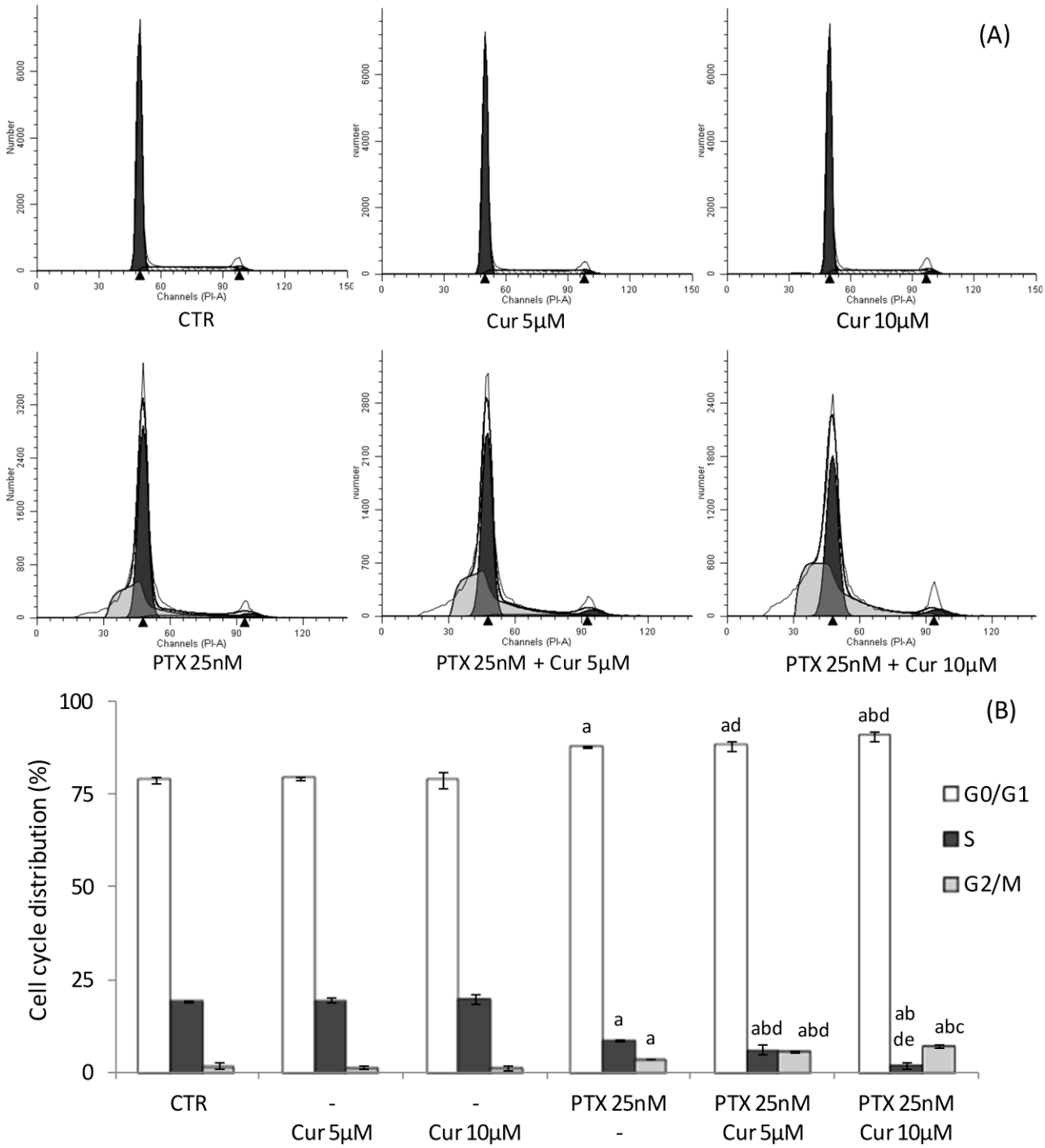

Fig. 3. Cell cycle analysis – (A) Cell cycle distribution in G0/G1, S and G2/M was measured in C6 glioma cells treated for 48 h with PTX (25 nM), Cur (5-10 µM) or with a combination of Cur (5-10 µM) and PTX (25 nM). (B) Percentage of cells in each phase. Data are reported as mean ± SD of three separate experiments. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs 25 nM PTX; [c]$p < 0.05$ vs 5 µM Cur; [d]$p < 0.05$ vs Cur (5 and 10 µM); [e]$p < 0.05$ vs 25 nM PTX plus 5 µM Cur.

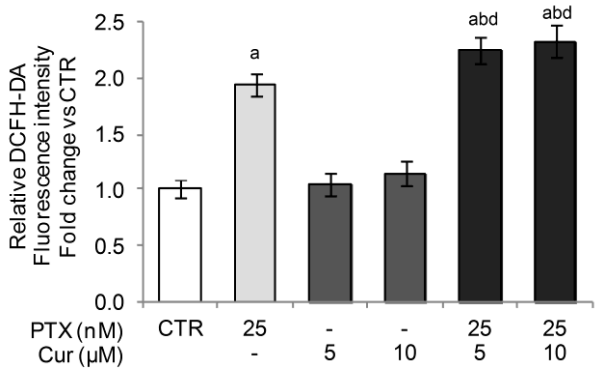

Fig. 4. Intracellular RS production – C6 glioma cells were treated for 48 h with PTX (25 nM), Cur (5-10 μM) or with a combination of Cur and PTX. Results are expressed as relative DCFH-DA fluorescent intensity and are reported as mean ± SD of three separate experiments. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs 25 nM PTX; [d]$p < 0.05$ vs Cur (5 and 10 μM).

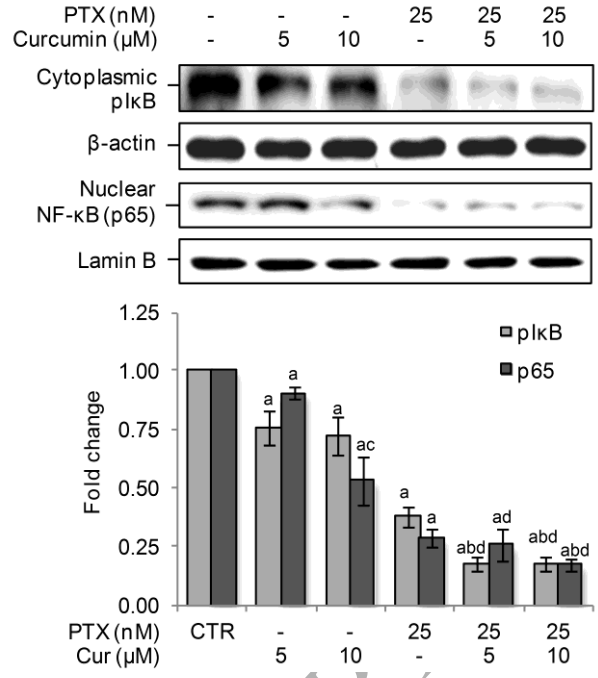

Fig. 5. Effects on NF-κB signaling pathway – C6 glioma cells were treated for 48 h with PTX (25 nM), Cur (5-10 μM) or with a combination of Cur (5-10 μM) and PTX (25 nM). Results by densitometry, normalized to the corresponding β-actin (pIκB) or Lamin B (NF-κB) value, are reported as fold change against CTR and expressed as mean ± SD of three separate experiments. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs 25 nM PTX; [c]$p < 0.05$ vs 5 μM Cur; [d]$p < 0.05$ vs Cur (5 and 10 μM).

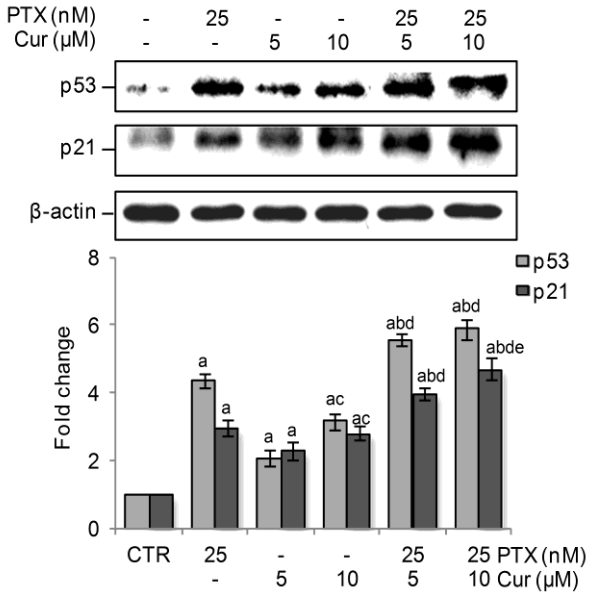

Fig. 6. Effects on p53 and p21$^{Waf1/Cip1}$ protein levels – C6 glioma cells were treated for 48 h with PTX (25 nM), Cur (5-10 μM) or with a combination of Cur (5-10 μM) and PTX (25 nM). Results by densitometry, normalized to the corresponding β-actin value, are reported as fold change against CTR and expressed as mean ± SD of three separate experiments. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs 25 nM PTX; [c]$p < 0.05$ vs 5 μM Cur; [d]$p < 0.05$ vs Cur (5 and 10 μM); [e]$p < 0.05$ vs 25 nM PTX plus 5 μM Cur.

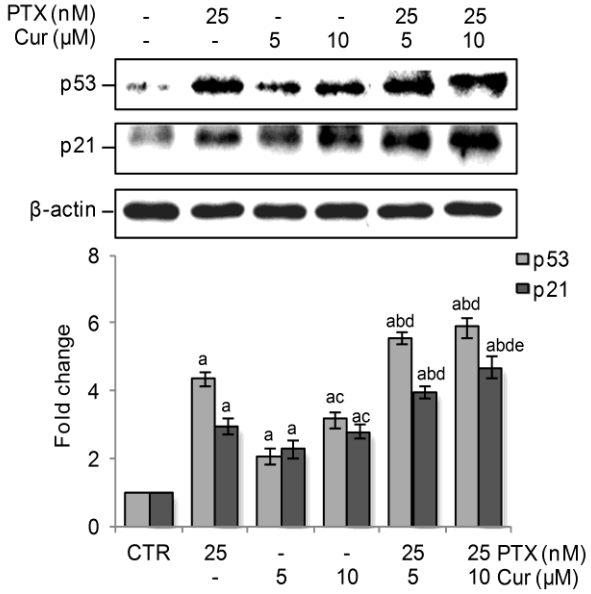

Fig. 7. Effects on caspase-3 (A, B) and Bcl-2 (A, C) activation – C6 glioma cells were treated for 48 h with PTX (25 nM), Cur (5-10 μM) or with a combination of Cur (5-10 μM) and PTX (25 nM). Results by densitometry, normalized to the corresponding β-actin value, are reported as fold change against CTR and expressed as mean ± SD of three separate experiments. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs 25 nM PTX; [c]$p < 0.05$ vs 5 μM Cur; [d]$p < 0.05$ vs Cur (5 and 10 μM); [e]$p < 0.05$ vs 25 nM PTX plus 5 μM Cur.

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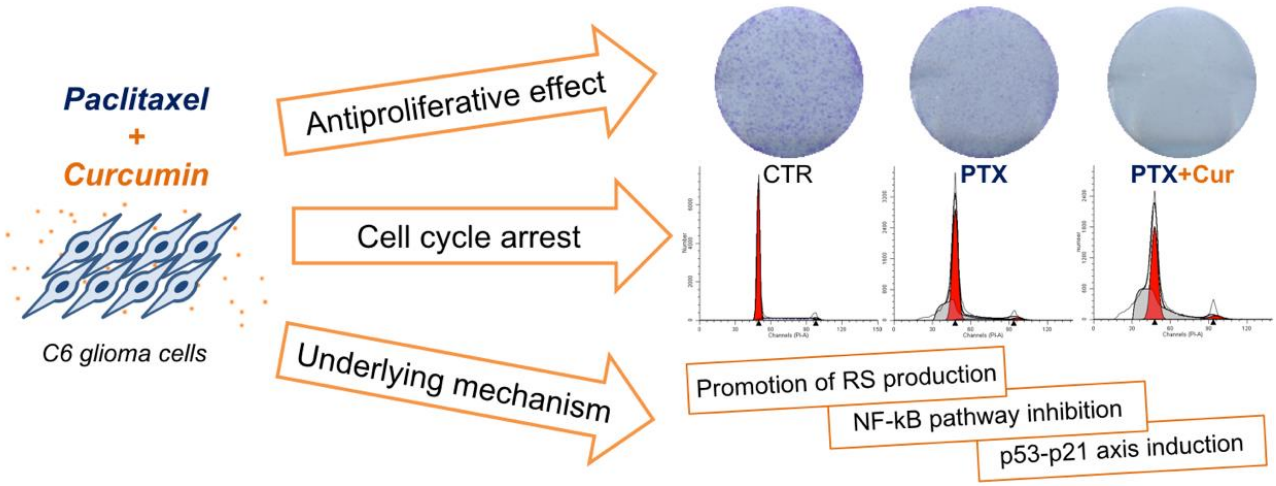

Graphical abstract