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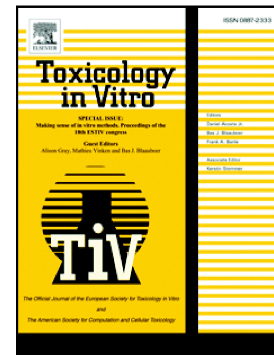

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Curcumin ameliorates the in vitro efficacy of carfilzomib in human multiple myeloma U266 cells targeting p53 and NF-κB pathways

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Abstract

Multiple myeloma (MM) is a malignant B-cell neoplasm with accumulation of malignant plasma cells in bone marrow. Pharmacological therapy improves response frequency even if with various associated toxicities. Herein, we investigated if combination of curcumin with carfilzomib (CFZ) can induce a better cytotoxic effect on in vitro cultured U266 cells. Cell viability data showed that curcumin significantly ameliorates CFZ cytotoxic effect. Furthermore, curcumin alone did not affect proteasome at the tested dose, confirming the involvement of different mechanisms in the observed effects. U266 cells exposure to curcumin or CFZ increased reactive species (RS) levels, although their production did not appear further potentiated following drugs combination. Interestingly, NF-κB nuclear accumulation was reduced by treatment with CFZ or curcumin, and was more deeply decreased in cells treated with CFZ-curcumin combinations, very likely due to the different mechanisms through which they target NF-κB. Our results confirmed the induction of p53/p21 axis and G0/G1 cell cycle arrest in anticancer activities of both drugs, an effect more pronounced for the CFZ-curcumin tested combinations. Furthermore, curcumin addition enhanced CFZ proapoptotic effect. These findings evidence that curcumin can ameliorate CFZ efficacy, and lead us to hypothesize that this effect might be useful to optimize CFZ therapy in MM patients.

Keywords: myeloma; curcumin; carfilzomib; NF-κB; p53; proteasome inhibitor.

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1. Introduction

Multiple myeloma (MM) is a malignant B-cell neoplasm characterized by the accumulation of malignant plasma cells in the bone marrow. The five-year survival rate for MM patients is around 40% and, to date, MM remains incurable. The therapy, represented by high dose chemotherapy with stem cell transplantation, improves the response frequency in MM patients even if it tends to have associated toxicities (Salem et al., 2013). The glucocorticoid analog dexamethasone and the proteasome-inhibitor drug bortezomib (BTZ) are considered the most effective treatments for MM (Palumbo and Anderson, 2011).

The ubiquitin-proteasome pathway is essential for many fundamental cellular processes, including the cell cycle, apoptosis, angiogenesis, and differentiation (Chen and Dou, 2010). It has been shown that inhibition of chymotrypsin-like activity in cancer cells is a solid stimulus that induces apoptosis (Chen and Dou, 2010). BTZ, the first US Food and Drug Administration approved proteasome inhibitor, has shown significant success; however about 60% of patients do not respond to BTZ due to the emergence of resistance (Merchionne et al., 2007). Recently, carfilzomib (CFZ), a tetrapeptide epoxyketone-based irreversible proteasome inhibitor, obtained responses in BTZ-resistant MM patients (Kuhn et al., 2007; Siegel et al., 2013).

However, cancer is so complex and refractory that the efficacy of monotherapy is usually limited in clinic. With the increased understanding of the mechanisms underlying the compromised therapeutic efficacy of monotherapy, drug combination therapy or drug cocktail therapy is extensively exploited and increasingly becoming the standard practice to combat the cancer. The principle underlying rational combined pharmacological strategies is to maximize efficacy and overcome treatment resistance by utilizing drugs with known efficacy, different mechanisms of action, and minimally overlapping toxicities. Through utilization of agents in combination, tumor biology can be exploited using a number of strategies, including

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molecular targeted therapies acting at multiple levels on a single pathway, and employment of agents with different mechanisms of actions against the same target, as well as strategies exploiting multiple targets on parallel pathways.

Thus, in recent years, new drugs or drug combinations targeting the p53 and NF-κB pathways to combat cancer have been evolving as an attractive strategy (Dey et al., 2008) which could have remarkable therapeutic potential also in MM. In fact , in hematologic malignancies, mainly lymphoid neoplasm, NF-κB signaling activation has been extensively described (Matthews et al., 2016). NF-κB is a transcription factor which modulates protein expression mediating cell cycle/proliferation, apoptosis, and cytokine secretion (Karin and Greten, 2005). It is constitutively present in the cytosol in the inactive form bound to the inhibitor of κBα (IκB). After activation, IκBα is phosphorylated by IκB kinases (IKKs), followed by its proteasomal degradation, thereby allowing nuclear translocation of NF-κB (Karin and Greten, 2005). The interest of the MM field for this signaling pathway is further increased by the attracting reports that observed NF-κB overactivation in virtually all primary MM patient samples and its involvement in chemoresistance (Markovina et al., 2010). Furthermore, NF-κB exerts its function mainly as an antagonist to p53 transactivation, a key tumor suppressor protein. The "guardian of the genome" p53, one of the most intensively studied tumour suppressors over the past two decades, is a DNA-binding transcription factor that induces activation of the intrinsic apoptotic pathway (Yu et al., 2016). So, given the immense relevance that the p53 and NF-κB pathways have in cellular physiology, it is not surprising that crosstalk between these two transcriptional regulatory networks has been identified and several studies have focused on the crucial reciprocal control of the p53 and the NF-κB pathways especially in human cancer, in which inactivation of p53 and hyperactivation of NF-κB is a common occurrence.

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One obvious way of hindering IκB degradation and thus avoiding nuclear NF-kB accumulation is proteasomal inhibition. Proteasome inhibitors are known to halt cell cycle progression and to induce apoptosis, both phenomena observed even more dramatically in malignant cells. Apart from affecting NF-kB, these inhibitors are likely to exert their effect via stabilizing cell cycle inhibitory proteins like p53 (Amit and Ben-Neriah, 2003; de la Puente et al., 2014).

On the other hand, curcumin, a phenolic compound isolated from the plant *Curcuma longa*, has been discovered to have chemopreventive action for an extensive type of tumors like colon, breast, lung, and esophagus (Aggarwal et al., 2003), modulating several transcription factors including NF-κB (Kasi et al., 2016). For example, curcumin down-regulates constitutive NF-κB activation in human MM cells and in CD138+ cells from MM patients, leading to proliferation suppression and apoptosis induction (Bharti et al., 2003; Bharti et al., 2004b). There is evidence that NF-κB, as a consequence of the treatment with curcumin, has a tendency to maintain bonding with IκB, since curcumin impedes the phosphorylation and degradation of IκBα and has been shown to specifically target IKKs (Kasi et al., 2016). Interestingly new data indicates that a better therapeutic effect may be attained when curcumin or its novel analogs are used in combination with proteosome inhibitors; these effects were observed on different MM cell lines (such as U266, H929, and Arp) treated with BTZ and appeared exerted through controlling NF-κB and Bcl-2 protein families expression (Bai and Zhang, 2012; Mujtaba et al., 2012; Park et al., 2008; Sung et al., 2009). This could be useful in the treatment of refractory malignancies. Finally, curcumin appeared able to potentiate the action of BTZ in an in vivo xenograft MM model in nude mice subcutaneously injected with U266 cells (Sung et al., 2009). However, there are no data about the toxicity of curcumin combined with the second-generation proteasome inhibitor CFZ on myeloma cells.

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In the present paper, we investigated if curcumin could ameliorate the cytotoxic effect of the second-generation proteasome inhibitor CFZ on in vitro cultured MM cells, when the two drugs are used in combination, due to their capability to modulate the p53-p21 and NF-κB pathways through different mechanisms.

2. Materials and Methods

2.1 Reagents

CFZ was supplied from LC Laboratories (Woburn, MA, USA) (>99% purity). Curcumin was supplied from Cayman Chemical (Ann Arbor, MI, USA) (≥ 90% purity). Antibodies, unless otherwise specified, were purchased from Cell Signaling (Beverly, MA, USA). All other reagents, unless otherwise specified, were purchased from Sigma Aldrich (Milan, Italy).

2.2 Cell culture and treatments

The U266B1 [U266] (ATCC® TIB-196™) cell line and the MM.1R (ATCC® CRL-2975™) cell line were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The U266B1 is a human mammalian cell line isolated from the peripheral blood of a 53-years-old male patient suffering from an IgE myeloma and used for studies about multiple myeloma (Nilsson et al., 1970). The MM.1R is a resistant variant of MM.1 cells, isolated from the original MM.1 culture based on their lack of responsiveness to cytolysis induced by the synthetic glucocorticoid (GC) dexamethasone, since it does not express the GC receptor. MM.1R is a mixed cell culture growing both as a lightly attached monolayer and in suspension. Subcultures were prepared by scraping the adherent cells into the medium containing the floating cells, collecting the cells by centrifugation, resuspending the cell pellet in fresh medium, and dispensing it into new flasks. U266B1 is a suspension cell culture;

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subcultures were prepared collecting cells by centrifugation, resuspending the cell pellet in fresh medium, and dispensing into new flasks.

Both cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine. Cells were maintained in an incubator with humidified atmosphere containing 5% CO_2 at 37°C. For all the experiments, CFZ and curcumin were always freshly dissolved in DMSO and water respectively. The final concentration of DMSO in the culture medium during the different treatments was always maintained at 0.1% v/v.

2.3 Cytotoxicity assays

The cytotoxic effect of CFZ and curcumin on U266 and MM.1R cells was measured using the trypan blue dye exclusion assay (Cimino et al., 2014). Cells were plated in 24-wells cell plates (initial density $5x10^5$ cells/well), and, after 24 hrs, were treated with CFZ (1-15 nM), curcumin (1-20 μM) or with a combination of curcumin (5 μM) and CFZ (1-10 nM) for 48 hrs. Results are reported as percentage of viable cells calculated from the number of viable cells in treated samples vs control. The 50% lethal concentration (LC_{50}), defined as the concentration needed to kill 50% of the treated cells in comparison with untreated control cells, and 90% confidence limits (C.L.) were calculated using the Litchfield and Wilcoxon test. The 48 hrs exposure time was selected because in a preliminary set of experiments 24 hrs exposure to curcumin or CFZ alone induced only a moderate cytotoxic effect (data not shown).

2.4 Cell protein expression and reactive species production

Cell protein expression and reactive species production were studied in U266 cells exposed to CFZ and curcumin alone or in combination. The doses of CFZ (2.5-5 nM) and curcumin (5

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μM) used throughout the study were chosen since they are able, if used in combination, to give an additive cytotoxic effect as calculated by using the Chou-Talalay combination index method (Chou, 2010). We have chosen to carry out the following experiments only on U266 cells for two reasons. First, cells with primary resistance (such as the MM.1R) or acquired resistance to drugs present biochemical adaptation phenomena (Kuhn et al., 2007; Salem et al., 2015; Soriano et al., 2016) that can very likely influence the response also to other pharmacological treatments. Secondly, U266 cells are characterized, in comparison with other cell lines such as MM.1S, by an overexpression of antioxidant systems (Salem et al., 2015) that makes them more resistant to treatment with molecules whose action mechanism is related to the formation of reactive species (as in the case of curcumin and CFZ, see findings reported in fig. 3); thus, using cells characterized by a lower intrinsic oxidative status reduces the possibility that deeper cytotoxic effects might be observed following exposure to a drug combination only as a consequence of reactive species accumulation.

2.4.1 Immunoblotting

Following appropriate treatment, cells were lysed in non-denaturing buffer (10 mM Tris HCl, 150mM NaCl, 1% Triton X-100, 5mM $EDTANa_2$, 1mM DTT, and protease inhibitors). For ubiquitylation studies cellular proteins were added with 1% SDS, and then boiled in order to inactivate deubiquitylases (Emmerich and Cohen, 2015). For nuclear proteins, cells were lysed at 2°C in a hypotonic buffer (10mM Hepes, 1.5mM $MgCl_2$, 10mM KCl, and 5% glycerol, 1mM DTT, and protease inhibitors), and treated with 0.65% Igepal for 5 min. Nuclei were recovered by centrifugation at 20.000×g at 4°C, lysed with a hypertonic buffer (20mM Hepes, 400mM NaCl, 1mM $MgCl_2$, 0.1mM EDTA, 1mM EGTA, 10% glycerol, 1mM DTT, and protease inhibitors), and stored at −70°C until use. Protein concentration in lysates was determined using Bradford reagent (Bradford, 1976).

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Forty µg of protein lysates were denatured in reducing sample buffer and subjected to SDS-PAGE. Separated proteins were transferred to nitrocellulose membrane (Hybond-P PVDF, Amersham Bioscience, Milan, Italy) and then blocked with 5% (w/v in TBS-T) nonfat milk overnight at 4°C. Membranes were then probed with specific primary antibodies diluted in TBS-T: caspase-3 rabbit mAb (1:1.500), $p21^{Waf1/Cip1}$ rabbit mAb (1:1.000), phospho-p53 rabbit mAb (1:1.500), NF-κB p65 rabbit mAb (1:1.000), ubiquitin rabbit pAb (1:2.000), β-Actin rabbit mAb (1:6.000), lamin-B mouse mAb (1:700); followed by secondary antibody HRP labeled goat anti-rabbit Ig (1:5.000) or HRP labeled goat anti-mouse IgM (1:10.000); and visualized with an ECL plus detection system (Amersham Biosciences, Milan, Italy). Densitometry was performed using ImageJ software (https://imagej.nih.gov/ij/).

2.4.2 Intracellular reactive species measurement by DCFH-DA assay

Intracellular reactive species (RS) were measured by the oxidation-sensitive fluorescent probe, dichloro-dihydro-fluorescein diacetate (DCFH-DA), as a modified method previously described by (Fratantonio et al., 2015). Cells exposed to 100 µM H_2O_2 for 1 h were used as positive internal controls. RS levels were measured as DCFH-DA relative fluorescence intensity and expressed as fold change vs control.

2.5 Cell cycle analysis

Cell cycle distribution was determined by DNA staining with BD Cycletest™ Plus DNA Kit. Briefly, 1×10^6 cells were plated in 6-wells cell plates, and, after 24 hrs, were incubated with CFZ, curcumin or with a combination of two compounds for 24 hrs. Cells were then washed in PBS and fixed in 70% cold ethanol overnight. Cells were collected and treated with BD Cycletest™ Plus DNA Kit. The PI fluorescence was then measured by flow cytometry (FACScan II, Becton Dickinson San Jose, CA, USA). A minimum of 20,000 cells were

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acquired per sample, and data were analyzed using the software Modfit 3. The percentage of cells in G0/G1, S and G2/M was determined from histograms of DNA content.

2.6 Statistical analysis

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

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3. Results

3.1 Cytotoxic effect of CFZ and curcumin

To evaluate the cytotoxic effect of CFZ and curcumin, U266 cells and MM.1R cells were treated with the two compounds alone or in combination. A 48 hr treatment with curcumin or CFZ alone induces a significant and dose dependent cell mortality, as shown by LC_{50} values reported in fig. 1 for U266 cells and in supplemental material for MM.1R cells (Fig. 1S). Curcumin 5 µM showed *per se* a moderate cytotoxicity but if used in combination with CFZ at various doses significantly improved the cytotoxic effect of this proteasome inhibitor on both MM cell lines (see fig. 1 and supplemental material). In particular the combination of CFZ with curcumin 5 µM, in fact, lowered CFZ LC_{50} on U266 cells from 5.2 nM to 3.3 nM (Figure 1).

3.2 Effects of CFZ and curcumin on accumulation of ubiquitinated proteins

CFZ irreversibly binds and selectively inhibits the chymotrypsin-like activity of the 20S proteasome leading to a more sustained proteasome inhibition (Demo et al., 2007). MM cells were particularly found to be susceptible to proteasome inhibition because they are proliferative, over-produce defective proteins that need to be degraded by the proteasome, and up-regulate signaling pathways dependent on the 26S proteasome (McBride and Ryan, 2013). As predictable, U266 treatment with CFZ caused an accumulation of ubiquitinated proteins (Figure 2). Conversely, western blot analysis revealed that 5 µM curcumin alone did not induce a significant ubiquitinated proteins accumulation in U266 cells. Furthermore, when in combination with CFZ 2.5 or 5 nM, 5 µM curcumin did not potentiate the ubiquitinated proteins accumulation induced by CFZ, so supporting that different mechanisms contribute to the cytotoxic effect observed in cells exposed to curcumin and CFZ together.

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3.3 Effects of CFZ and curcumin on intracellular RS production

Studies have shown that, if compared to normal cells, cancer cells can be rendered susceptible to reactive oxygen species (ROS)-mediated cytotoxicity as they are intrinsically under oxidative stress due to increased steady-state levels of $O_2^{\bullet-}$ and H_2O_2 from mitochondrial metabolism. Oxidative stress has been identified as an important marker in many cancers (Gangemi et al., 2012) and a mechanism of BTZ cytotoxicity in myeloma cells (Goel et al., 2012). Furthermore, co-treatment with the antioxidants rescued from BTZ-induced RS generation and cell death (Salem et al., 2013).

Our data showed that CFZ and curcumin were able to increase RS levels in U266 cells, although the effects were not dose-dependent (Figure 3). Furthermore, the CFZ-curcumin combinations were unable to increase RS levels compared to single drug treatment. These results confirm that RS increase is important for cell cytotoxicity but is not directly related to the deeper cell death observed when the CFZ-curcumin combination is used.

3.4 Effects of CFZ and curcumin on NF-κB signaling pathway

Patient-derived MM tumor cells and bone marrow stromal cells present upregulated NF-κB levels relative to normal cells, and intrinsic activation of NF-κB is associated with growth/survival of MM cells (Ma et al., 2003). Findings indicate that NF-κB is a key regulator of growth and survival of MM cells in the bone marrow milieu, thus the rationale of several therapeutic strategies is to down-regulate NF-κB activation, thereby enhancing the cytotoxic effects of chemotherapy (Chauhan and Anderson, 2003). While CFZ in MM cells inhibits NF-κB activity by blocking proteasomal degradation of IκBα, curcumin-induced down-regulation of NF-κB nuclear accumulation seems due to the inhibition of IκBα phosphorylation (Bharti et al., 2003). As shown in Figure 4, U266 treatment with CFZ or curcumin, at all the tested doses, decreased p65 nuclear levels compared to the basal control

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levels. Moreover, down-regulation of this signaling pathway was significantly stronger in cells treated with the CFZ-curcumin combinations then in those treated with one of the two compounds alone, probably targeting NF-κB activity by distinct mechanisms.

3.5 CFZ and curcumin modulate the p53-p21 axis in U266 cells

We wanted to investigate if CFZ and curcumin, alone or in combination, can induce the p53-p21 axis activity that, on turn, modulates the proapoptotic cellular response. The unlimited replication potential of cancer cells can be a result of the inactivation of tumor suppressor gene p53. The p53 regulates molecules involved in apoptotic pathways and cell cycle regulation. The cell cycle-related protein p21 (known as $p21^{Waf1/Cip1}$), a CDK2 and CDK1 inhibitory protein (Satyanarayana et al., 2008) transcriptionally regulated by p53, causes G1 cell cycle arrest (Waldman et al., 1995). In addition, p21 directly inhibits proliferative cell nuclear antigen (PCNA)-dependent DNA replication and modulates PCNA-dependent DNA repair system (Waga et al., 1994). Our results showed an upregulation of both p53 and p21 in U266 cells exposed to CFZ or curcumin (Figure 5 A and B). Moreover, the CFZ-curcumin tested combinations further increased both proteins, showing a deeper effect of these two compounds used together in cell growth arrest.

3.6 CFZ and curcumin induce cell cycle arrest

p53 influences proliferation by acting predominately in the G1 phase of the cell cycle progression by inducing expression of p21 and the consequent inhibition of CDKs. The results showed in figure 6 depict a representative profile of cell cycle distribution in U266 cells following different treatments. In the control group, cells without drug exposure presented 51.7% and 36.8% populations in G0/G1 and S phase, respectively. Treatment with CFZ caused a slight accumulation of the G0/G1 fraction (up to 61.1%; $p < 0.05$ vs CTR) and

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a reduction of S phase (up to 29.1%; $p < 0.05$ vs CTR) in a dose-dependent way. At the same extent, approximately 62.6% ($p < 0.05$ vs CTR) of the cells were arrested at the G0/G1 phase of the cell cycle, while 25.7% ($p < 0.05$ vs CTR) were at S phase, when treated with 5μM curcumin. Interestingly, CFZ-curcumin combinations further increased the G0/G1 fraction and reduced S phase up to 73.9% and 17.4% respectively ($p < 0.05$ vs CTR; $p < 0.05$ vs respective CFZ dose treatment), indicating that this drug association is able to better halt cells in G0/G1 phase.

3.7 CFZ and curcumin induce apoptosis in U266 cells

Caspase-3 is a cytosolic protein that exists normally as a higher molecular weight inactive precursor (pro-caspase-3). It is proteolytically cleaved into a low molecular weight active enzyme when cells undergo apoptosis (Shalini et al., 2015). To determine the proapoptotic effect of CFZ and curcumin we performed western blot analysis for verifying caspase-3 cleavage status in U266 cells treated with the two compounds alone (curcumin 5 μM; CFZ 2.5 or 5 nM) or in combination. Both CFZ and curcumin induced caspase-3 activation at the tested doses (Figure 7). Moreover, 5 μM curcumin addition significantly enhanced CFZ proapoptotic effect, with increased caspase-3 levels compared to CFZ or curcumin alone.

4. Discussions

MM is a B-cell malignant disorder characterized by clonal proliferation of plasma cells in the bone marrow and osteolytic bone lesions (Genadieva-Stavric et al., 2014). Although new therapeutic options, such as the second-generation selective proteasome inhibitor CFZ (Fostier et al., 2012) have been introduced and overall survival rate has improved in the management of MM, the disease remains incurable and almost all patients show disease relapse and develop drug resistance. So, combinational therapies with two or more agents are

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often required to improve the prognosis of MM patients. In fact, combinatorial regimens can optimize therapy efficiency, improve target selectivity, and minimize the development of cancer drug resistance. The search for effective chemopreventive agents derived from vegetable sources has identified several compounds with anti-tumor activity against many different cancers.

In this study, we aimed to evaluate the effects of curcumin, CFZ, and their combinations for their antiproliferative and cytotoxic effects on U266 myeloma cells. The combination of curcumin and CFZ was more effective than either agent alone and the cell viability data showed that a significantly deeper effect can be achieved combining 5 μM curcumin also with concentrations of CFZ able to induce a poor cytotoxic effect (around 25% reduction of cell viability, as observed for CFZ 2.5 nM). These results obtained in U266 cells were confirmed also by using dexamethasone-resistant myeloma MM.1R cells. Our findings lead us to speculate that curcumin could potentiate the clinical efficacy of CFZ, thus decreasing toxicity issues that are associated with the use of high-dose CFZ. Furthermore, these findings are in agreement with those reported by other Authors about the positive effect of the combination of BTZ and curcumin on MM cells (Bai and Zhang, 2012; Mujtaba et al., 2012; Park et al., 2008; Sung et al., 2009), leading us to hypothesize that synegizing effects of curcumin with proteasome inhibitors might be a general phenomenon.

To further investigate the molecular mechanisms by which the combined treatment of curcumin and CFZ results in a better inhibition of MM cell growth, we examined the proteasome inhibition in U266 cells treated with each drug alone or a combination of them. Our data demonstrated that, unlike CFZ, curcumin alone is unable to affect proteasome at the tested dose (5 μM) and it does not potentiate CFZ-induced proteasome inhibition, so confirming the involvement of different mechanisms in the observed effects following curcumin and CFZ cotreatment. Even if the tumor cellular proteasome has been proposed as a

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potential target also for curcumin (Yang et al., 2008), conflicting data was shown in literature probably due to the different concentrations used. In fact, curcumin was able to induce proteasome inhibition only at concentrations greater than 10 μM both in human colon carcinoma HCT-116 and SW480 metastatic cell lines (Milacic et al., 2008), and in MM U266 cells (Mujtaba et al., 2012).

Furthermore, increase in intracellular ROS can induce cancer cell cycle arrest, senescence and apoptosis and this can be achieved with several cancer chemotherapic drugs. In fact, our data demonstrated that U266 treatment with curcumin or CFZ increased RS levels when compared to controls. However, the cell RS production did not appear further potentiated when cells were exposed to the two drugs together. These results demonstrate that RS increase could be relevant for the antiproliferative effects of curcumin and CFZ but does not explain the better effect observed in cells treated with both drugs together.

To demonstrate if the anti-myeloma activity of both curcumin and CFZ is associated with their ability to inhibit the NF-κB signaling pathway, we investigated the effects of combined treatment with curcumin and CFZ on this transcription factor. The interest for this signaling pathway in MM therapy is further increased because the constitutive activation of NF-κB in tumor cells is quite higher than in normal cells (Bharti et al., 2004a). In particular, given that various processes may be involved in modulating the NF-κB pathway, the use of different inhibitors, acting on different steps of this pathway, could be relevant for a rational combination chemotherapy (Amit and Ben-Neriah, 2003).

We demonstrated that U266 treatment with CFZ or curcumin, at all the tested doses, decreased p65 nuclear levels compared to the basal control ones. This is in agreement with other findings from the literature, where CFZ is reported to inhibit NF-κB activity by blocking proteasomal degradation of IκBα (Guan et al., 2016); furthermore, it has been reported that curcumin can inhibit MM cell proliferation by reducing NF-κB activity (Allegra

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et al., 2016; Bharti et al., 2003). Interestingly we demonstrated that NF-κB nuclear accumulation was more deeply reduced in cells treated with the CFZ-curcumin combinations very likely due to the different mechanisms through which these two drugs target NF-κB. There is evidence that NF-κB represses p53 stability, so suppressing NF-κB and p53 the activities of each other (Dey et al., 2008; Yu et al., 2016). Recently there is increasing interest in identifying molecules acting simultaneously as activators of p53 and inhibitors of NF-κB, due to the crosstalk between these two critical pathways. So we have investigated if the cytotoxic effect observed in MM cells exposed to curcumin and CFZ may be related to a dual mechanism of activating p53 and repressing NF-κB. Many anticancer agents induce apoptosis and cell cycle arrest by modulating the tumor suppressor gene p53. In fact, a number of studies have raised the possibility that cells lacking p53 activity might be more readily resistant to cancer chemotherapy (Hassan et al., 2014). The p53 protein regulates molecules involved in apoptotic pathways and increases the amount of p21 protein, a cell cycle regulator that contributes to the arrest of cell in G1 phase (Warfel and El-Deiry, 2013). In this scenario, our results have confirmed the involvement of p53 pathway in anticancer activities of curcumin and CFZ in U266 cells. Both of them, in fact, induced p53 upregulation but this effect was more pronounced in the CFZ-curcumin tested combinations. Similarly, the effect of curcumin and CFZ on p21 upregulation was evident for single drug treatment and further increased by the associations. We also confirmed G0/G1 cell cycle arrest for curcumin and CFZ and a reduction of S phase in U266 cells. Interestingly, this effect was more prominent for the CFZ-curcumin combinations.

Finally, both CFZ and curcumin induced, at the tested doses, caspase-3 activation, a major executioner protease responsible for initiating the apoptotic program. Interestingly, 5 µM curcumin addition enhanced CFZ proapoptotic effect, with increased caspase-3 levels in comparison with those measured in cells exposed to CFZ or curcumin alone.

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In conclusion, these results suggest that curcumin can ameliorate the therapeutic efficacy of CFZ, and supply a mechanistic understanding for the antiproliferative effects of this drug combination involving activation of p53-p21 axis and NF-κB inhibition. The finding that curcumin suppresses NF-κB independently of the proteasome inhibition in U266 cells is of clinical significance, as curcumin and CFZ both modulate this signalling pathway but through different mechanisms, and represents a rational base for a potential use of these two agents in combination in treatment of MM patients.

It is well known that interactions between MM cells and bone marrow stromal cells (BMSCs) in bone marrow microenvironments have a fundamental role in modulating MM cell proliferation and resistance to anticancer drugs, through expression and release of mediators involved in MM cell growth and survival (Murray et al., 2014). BMSCs enhance viability of IL-6 dependent U266 cells since they release various factors (including IL-6) promoting MM cell growth and involved in JAK/STAT and MAPK-mediated pathways (Park et al., 2008). Curcumin ameliorated BTZ effect on U266 cells co-cultured with BMSCs (Park et al., 2008) acting on both U266 and BMSCs and so affecting their interactions. Thus, further experiments are warranted to investigate if also the combination of curcumin and CFZ may be useful to overcome the protective effect conferred by BMSCs in MM cells.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethics statement

This study is not reporting any experiment involving human subjects or animals.

Acknowledgements

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Figure captions

Figure 1. Cell viability and LC_{50} values at 48 hrs - U266 cells were treated for 48 hrs with CFZ (1-10 nM), curcumin (1-20 μM) or with a combination of curcumin (5 μM) and CFZ (1-10 nM). Viable cells were counted by trypan blue assay. Cultures treated with the vehicle alone were used as controls (CTR). Cell viability data represent percentage of viable cells in treated samples vs control. $^{a}p < 0.05$ vs lower curcumin doses; $^{b}p < 0.05$ vs lower CFZ doses; $^{c}p < 0.05$ vs lower CFZ doses in combination with 5 μM curcumin; $^{d}p < 0.05$ vs corresponding CFZ dose not in combination with curcumin; $^{e}p < 0.05$ vs 5 μM curcumin; $^{f}p < 0.05$ vs CFZ alone.

Figure 2. Accumulation of ubiquitinated (Ub_n) proteins – U266 cells were treated for 24hrs with CFZ (2.5 and 5 nM), curcumin (5μM), or with a combination of curcumin (5μM) and CFZ (2.5 and 5 nM). Cultures treated with the vehicle alone were used as controls. Reported image is representative of three independent experiments. β-actin was used as housekeeping protein.

Figure 3. **Intracellular reactive species production** – U266 cells were treated for 24 hrs with CFZ (2.5 and 5 nM), curcumin (5μM), or with a combination of curcumin (5μM) and CFZ (2.5 and 5 nM). Cultures treated with the vehicle alone were used as controls (CTR). Cells exposed to H_2O_2 for 1 h were used as positive control of increased intracellular RS level. 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 all treatments with CFZ and/or curcumin.

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Figure 4. Nuclear NF-kB (p65) – U266 cells were treated for 24hrs with CFZ (2.5 and 5 nM), curcumin (5µM), or with a combination of curcumin (5µM) and CFZ (2.5 and 5 nM). Cultures treated with the vehicle alone were used as controls (CTR). Results by densitometry are reported as fold change against control, expressed as mean ± SD of three separate experiments and normalized to the corresponding lamin b value. ^{a}p <0.05 vs CTR; ^{b}p <0.05 vs 2.5 nM CFZ; ^{c}p <0.05 vs 5 nM CFZ; ^{d}p <0.05 vs 5µM curcumin; ^{e}p <0.05 2.5nM CFZ + 5µM curcumin.

Figure 5. Effects on p53 (A) and p21$^{Waf1/Cip1}$ (B) protein levels – U266 cells were treated for 24hrs with CFZ (2.5 and 5 nM), curcumin (5µM), or with a combination of curcumin (5µM) and CFZ (2.5 and 5 nM). Cultures treated with the vehicle alone were used as controls (CTR). Results by densitometry are reported as fold change against control, expressed as mean ± SD of three separate experiments and normalized to the corresponding β-actin value. ^{a}p <0.05 vs CTR; ^{b}p <0.05 vs 2.5nM CFZ; ^{c}p <0.05 vs 5nM CFZ; ^{d}p <0.05 vs 5µM curcumin.

Figure 6. Cell cycle analysis – Cell cycle distribution in G0/G1, S and G2/M was measured in U266 cells treated for 24hrs with CFZ (2.5 and 5 nM), curcumin (5µM), or with a combination of curcumin (5µM) and CFZ (2.5 and 5 nM). Cultures treated with the vehicle alone were used as controls (CTR). The percentage of cells in each phase is provided on each cell cycle figure. The data are reported as mean ± SD of three separate experiments.

Figure 7. Effects on caspase-3 activation – U266 cells were treated for 24 hrs with CFZ (2.5 and 5 nM), curcumin (5 µM), or with a combination of curcumin (5 µM) and CFZ (2.5 and 5 nM). Cultures treated with the vehicle alone were used as controls (CTR). Results by densitometry are reported as fold change against control, expressed as mean ± SD of three

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separate experiments and normalized to the corresponding β-actin value. [a]$p < 0.05$ vs CTR; [b]$p < 0.05$ vs 2.5 nM CFZ; [c]$p < 0.05$ vs 5 nM CFZ; [d]$p < 0.05$ vs 5 μM curcumin.

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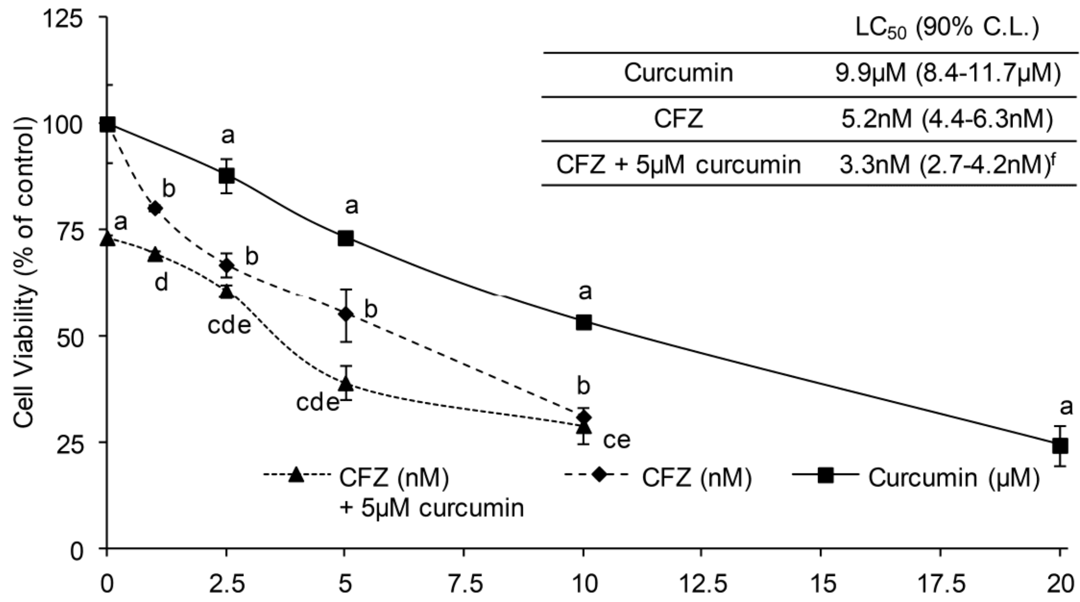

Fig. 1

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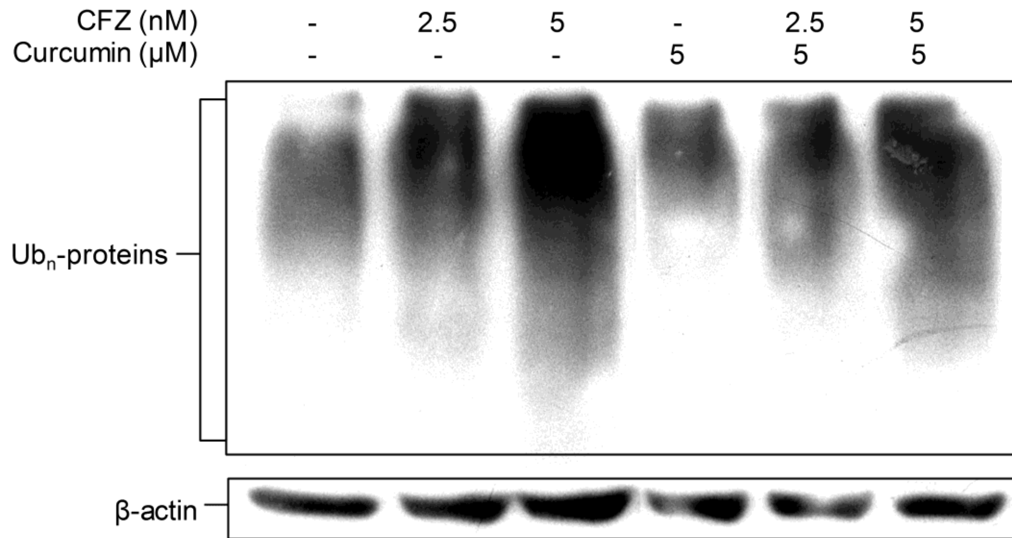

Fig. 2

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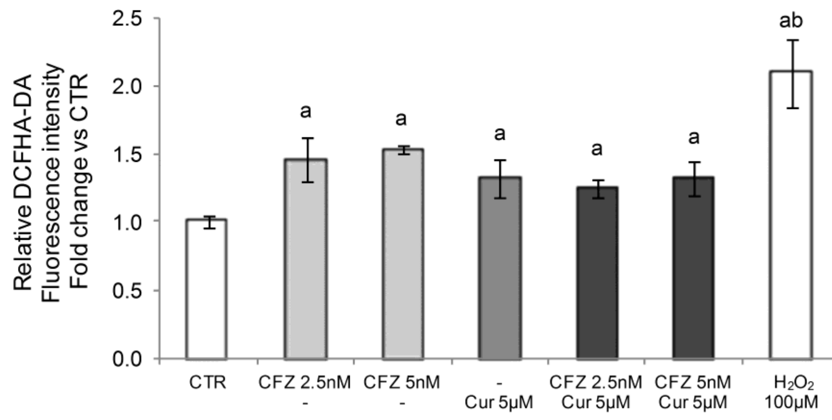

Fig. 3

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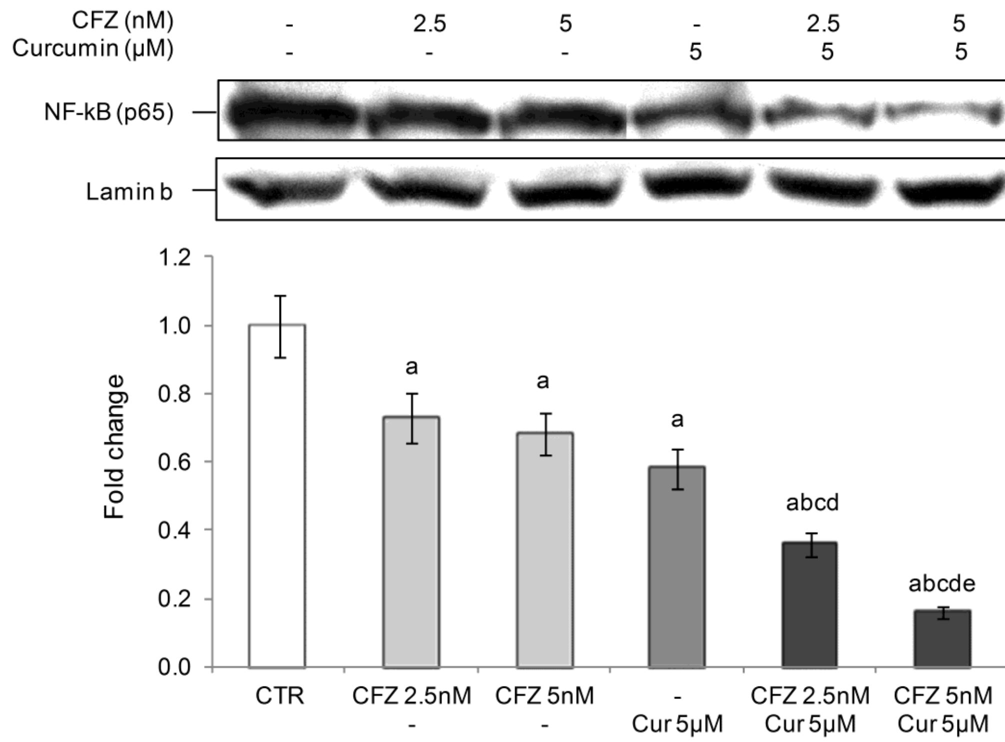

Fig. 4

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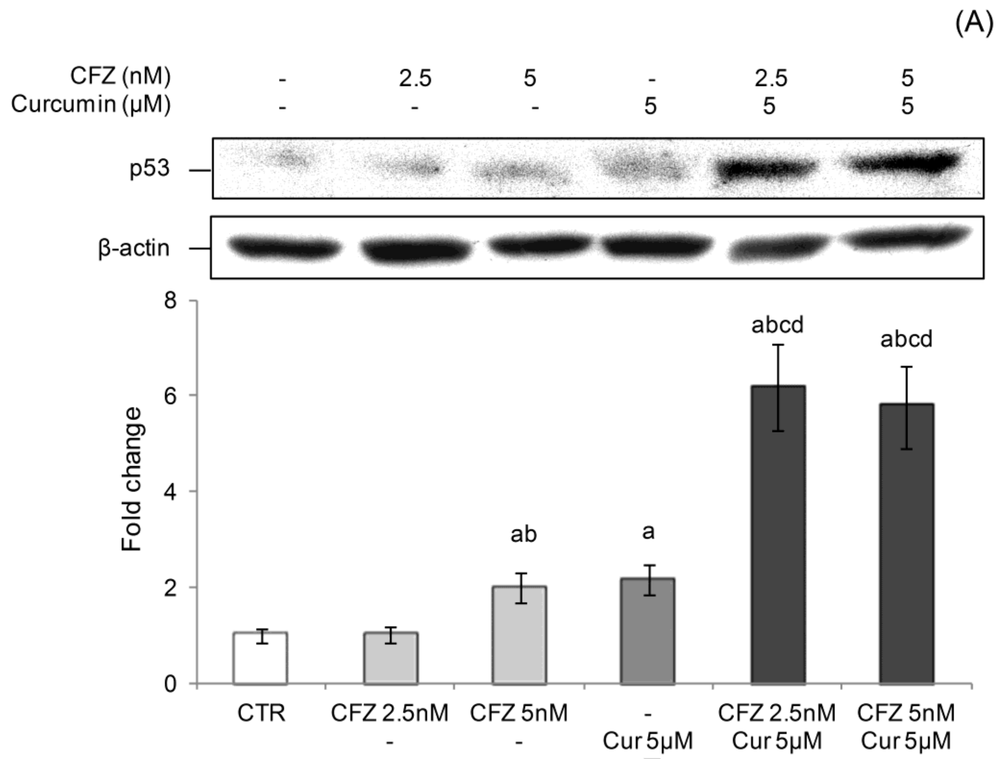

Fig. 5A

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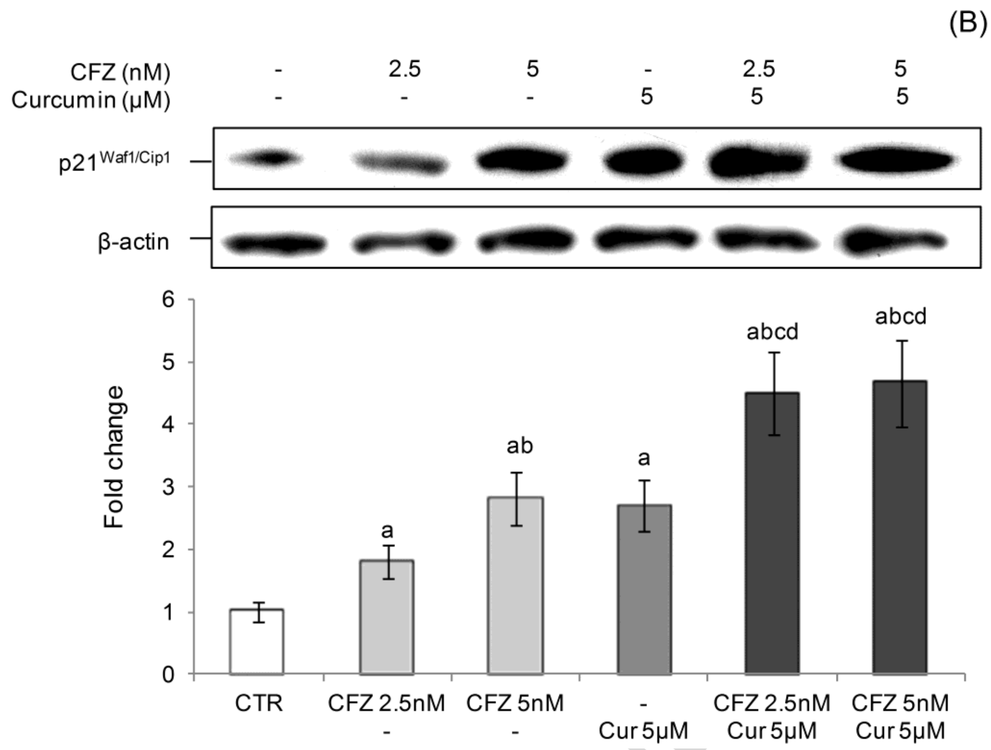

Fig. 5B

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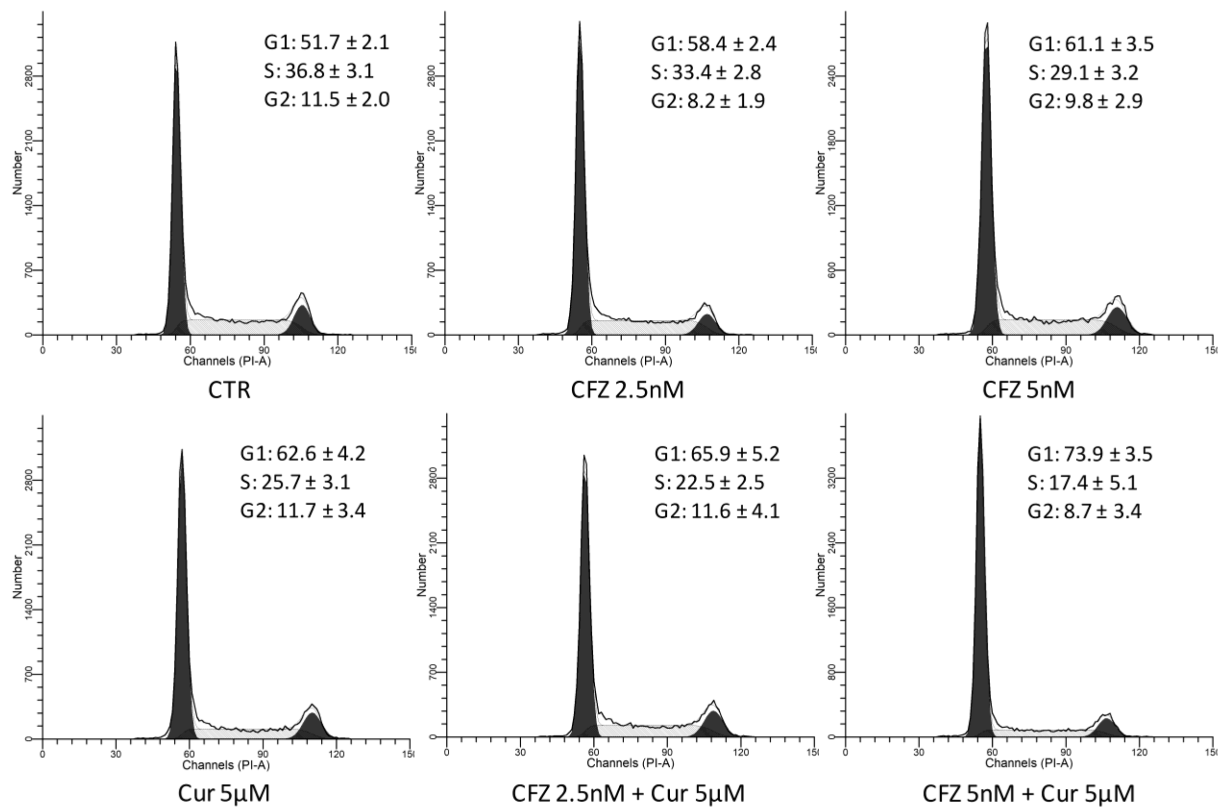

Fig. 6

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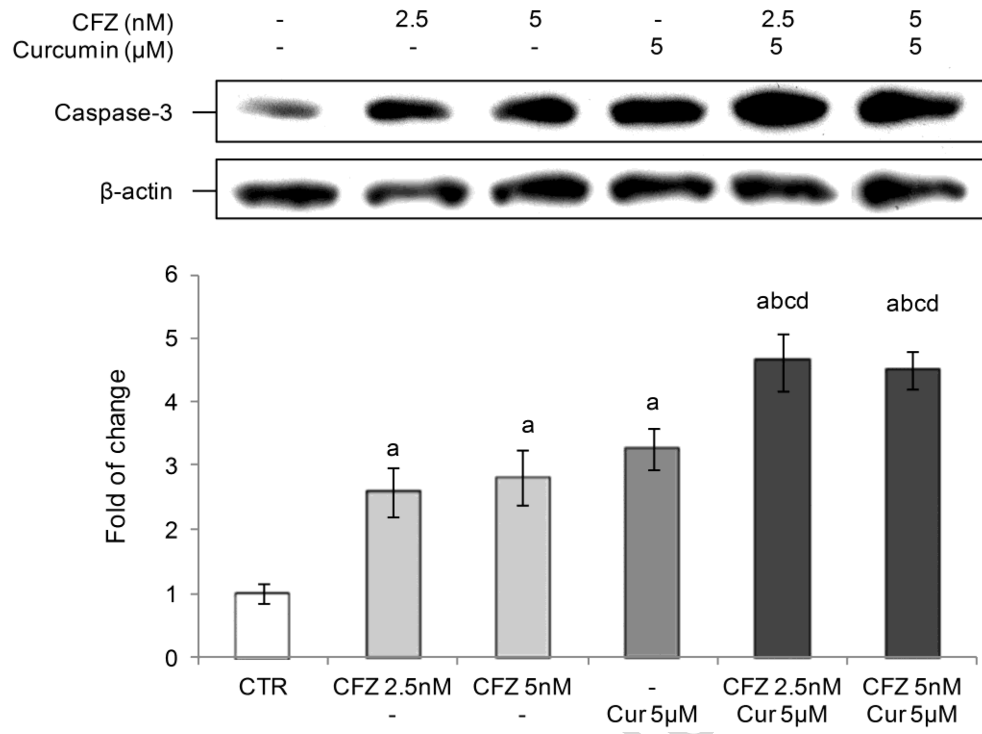

Fig. 7

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Highlights

- Curcumin significantly ameliorates in vitro cytotoxic of CFZ on U266 cells.
- Curcumin enhances CFZ induction of p53/p21 axis and apoptosis.
- CFZ-curcumin combination deeply reduces NF-κB nuclear accumulation in U266 cells.
- Curcumin suppresses NF-κB independently of the proteasome inhibition in U266 cells.
- Curcumin and CFZ modulate NF-κB signalling pathway through different mechanisms.

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