The synthetic flavagline FL3 spares normal human skin cells from its cytotoxic effect via an activation of Bad

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Abstract

The molecular pathways by which flavagline derivatives exert their cytotoxicity against various cancer cell types are well documented, while the mechanisms that prevent their cytotoxic effects on normal cells still have to be clarified. Here we provide the underlying molecular events by which normal skin cells remain unaffected after exposure to the synthetic flavagline FL3. Indeed, the anticancer agent fails to trigger apoptosis of healthy cells and is unable to induce the depolarization of their mitochondrial membrane and the cytosolic release of cytochrome C, in contrast to what is observed for cancer cells. Most importantly, FL3 specifically induces in normal cells, but not in malignant cells, an activation of Bad, without significant mitochondrial and cytosolic redistribution of Bax or Bcl-2. Moreover, gene knockdown of Bad sensitizes the normal fibroblastic cells to FL3 and induces a caspase-3 dependent apoptosis. Bad activation, known to promote survival and block apoptosis, explain therefore the lack of cytotoxicity of FL3 on normal skin cells. Finally, these findings provide new insights into the molecular resistance mechanisms of resistance of healthy cells against FL3 cytotoxicity and identify it as a promising anticancer drug.

Keywords	flavagline; normal skin cells; cytotoxicity; resistance; Bad phosphorylation
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Dear Editor,

Please find enclosed our manuscript entitled « The synthetic flavagline FL3 spares normal human skin cells from its cytotoxic effect via an activation of Bad » by Fathi Emhemmed, Sarah Ali Azouaou, Sarah Hassan, Ray Lefevbre, Laurent Désaubry, Christian D. Muller and Guy Fuhrmann, which we would like to submit for publication in « Toxicology in Vitro » as research article.

Our manuscript provides molecular evidence that a flavagline derivative, namely FL3, selectively kills cancer cells, but spares normal cells. This selectivity involves of Bad-dependent process which triggers a protective effect of the pharmacological agent on normal cells against its cytotoxic activity. In regard to the literature, our results show that this compound could therefore be a promising drug for the treatment of cancer since it is able to target the oncogenic process without affecting normal cells.

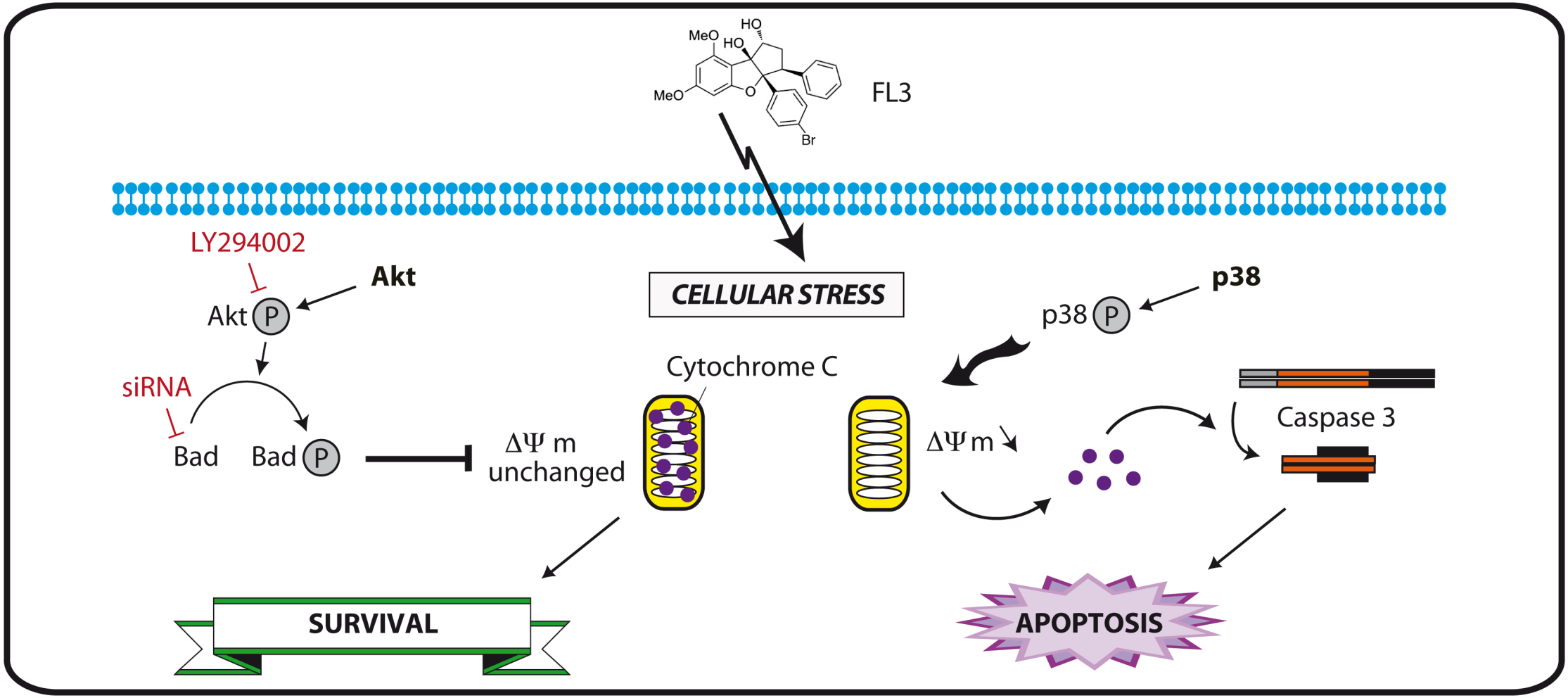
All the named authors state that they have agreed to the submission of this manuscript. All the named authors have participated in the study to a sufficient extent to be named as authors. Due care has been taken to ensure the integrity of the work. No part of this paper has been published or submitted elsewhere. No conflict of interest exits in the submission of this manuscript.

We appreciate your consideration of our manuscript, and we look forward to receiving comments from the reviewers.

Yours sincerely,

Fathi EMHEMMED , Ph D

- The synthetic flavagline FL3 selectively kills cancer cells, without having any effect on normal cells
- FL3 specifically induces the activation of Bcl-2-associated death promoter protein (Bad) in normal skin cells
- Knock-down of Bad removes the protection of normal skin cells against the cytotoxic effect of FL3
- The resistance of normal cells against FL3 cytotoxicity identifies it as a promising drug for an elective anticancer therapy



PRO-SURVIVAL AND PRO-APOPTOTIC SIGNALING EVENTS INDUCED BY FLAVAGLINE (ie FL3) IN NORMAL CELL

The synthetic flavagline FL3 spares normal human skin cells from its cytotoxic effect via an activation of Bad

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ABSTRACT

The molecular pathways by which flavagline derivatives exert their cytotoxicity against various cancer cell types are well documented, while the mechanisms that prevent their cytotoxic effects on normal cells still have to be clarified. Here we provide the underlying molecular events by which normal skin cells remain unaffected after exposure to the synthetic flavagline FL3. Indeed, the anticancer agent fails to trigger apoptosis of healthy cells and is unable to induce the depolarization of their mitochondrial membrane and the cytosolic release of cytochrome C, in contrast to what is observed for cancer cells. Most importantly, FL3 specifically induces in normal cells, but not in malignant cells, an activation of Bad, without significant mitochondrial and cytosolic redistribution of Bax or Bcl-2. Moreover, gene knockdown of Bad sensitizes the normal fibroblastic cells to FL3 and induces a caspase-3 dependent apoptosis. Bad activation, known to promote survival and block apoptosis, explain therefore the lack of cytotoxicity of FL3 on normal skin cells. Finally, these findings provide new insights into the molecular resistance mechanisms of resistance of healthy cells against FL3 cytotoxicity and identify it as a promising anticancer drug.

Keywords: flavagline; normal skin cells; cytotoxicity; resistance; Bad phosphorylation

1. Introduction

Flavaglines, such as rocaglamide, aglaiastatin or silvestrol, are natural compounds of plants from the genus *Aglaia* (Hausott et al., 2004). They show, at nanomolar concentrations, a cytotoxicity against many cancer cells, either *in vitro* or *in vivo* (Ebada et al., 2011; Ribeiro et al., 2012). Interestingly, flavaglines and their synthetic derivatives kill cancer cells, with no obvious toxicity on various normal cells (Thuaud et al., 2009, 2011). Accordingly, we recently observed that the synthetic flavagline FL3 has only a mild effect on the proliferation and viability of fibroblasts (Emhemmed et al., 2014), in contrast to teratocarcinomal cells which are killed through the activation of a pro-apoptotic process which is p38 MAPK-dependent. The activity of this signaling pathway is characterized by a disruption of the mitochondrial membrane, leading to release of cytochrome C to the cytosol which, in turn, activates caspase-3 (Porras and Guerrero, 2011). Accordingly, we observed that the phosphorylation of p38 MAPK in FL3-treated teratocarcinomal cells leads to the cleavage of caspase-3 and consequently to cell death. In agreement with our results, it has also been shown that rocaglamide or aglaiastatin induces in cancer cells a pro-apoptotic process involving the activation of p38 MAPK which recruits subsequently specific members of the Bcl-2 family (Hausott et al., 2004; Zhu et al., 2007).

The mitochondrial membrane permeabilization and depolarization occurs by homoligomerization of pro-apoptotic effector proteins such as Bcl2-associated X protein (Bax) or Bcl2-antagonist/killer protein (Bak); this leads to the formation of an oligomeric pore in the mitochondria which allows an escape of cytochrome

C, activation of the caspase cascade and induction of apoptosis. However, oligomerization requires association with BH3-only proteins, such as Bcl2-associated death promoter protein (Bad) which acts indirectly as pro-apoptotic factor, by displacing and freeing Bax from antiapoptotic proteins Bcl-2/Bcl-xL. However, Bad phosphorylation leads to the formation of a complex with 14-3-3 protein in the cytosol, thereby remaining Bax associated with the antiapoptotic proteins Bcl2/Bcl-xL and inhibiting the pro-apoptotic process (Dai et al., 2016). In this point of view, it has been shown that stably expressing Bcl-2 or Bax knockout cells are resistant to rocaglamide or silvestrol-induced apoptosis (Lindqvist et al., 2012; Zhu et al., 2007). This suggests that modifications in the activity of specific members of the Bcl-2 network could account for the inability of flavaglines to kill either cancer or normal cells.

The present work aims to study more in depth the molecular mechanisms by which FL3 spares healthy cells from its cytotoxic activity. Here we show that the drug specifically induces in fibroblasts a pro-survival mitochondrial process involving the activation of Bad. Forced repression of Bad phosphorylation causes cell death in FL3-treated fibroblasts. Our work therefore provides the mechanistic events by which normal cells actively escape from the selective pro-apoptotic activity of the anticancer compound FL3.

2. Materials and methods

2.1. Cell culture and treatment

Human BJ foreskin fibroblast (CRL-2522), HGF-1 gingival fibroblast (CRL-2014), NT2/D1 teratocarcinoma (CRL-1973), A375 melanoma (CRL-1619) and MCF-7 breast cancer (HTB-22) cell lines were purchased from ATCC (LGC Standards, Molsheim, France). Human HaCaT keratinocyte cell line was purchased from AddexBio (San Diego, CA, USA). All cell lines were cultured in high-glucose Dulbecco's Modified Eagle's medium (4.5 g glucose/l) (Sigma-Aldrich, Saint-Quentin Fallavier, France), supplemented with 10% (v/v) fetal bovine serum (Lonza, Verviers, Belgium), 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich). Cell culture was maintained in humidified incubator at 37°C in 5% CO2 atmosphere. FL3 was synthesized, diluted at 10 mM in dimethyl sulfoxide (DMSO) and added to the cell culture medium at a working concentration of 2000 nM, as previously described (Emhemmed et al., 2014, 2017). To avoid cytotoxicity or cell differentiation, DMSO concentration never exceeded 0.02% (v/v) in culture medium. The inhibitor of Akt phosphorylation LY294002 (Sigma-Aldrich) was used at a final concentration of 40 µM, according to the manufacturer's instructions.

2.2. siRNA transient transfection

Cells at a density of 2 x 10^5 cells/well were seeded in 6-well plates, cultured with free antibiotic Opti-MEM reduced serum (Thermo Fisher Scientific, Villebon sur Yvette, France) and then transfected with 120 nM of Bad small interfering RNA (siRNA) (Cell Signaling Technology, Danvers, MA, USA) in presence of INTERFERin (Polyplus-transfection, Illkirch, France), following the manufacturer's recommendations. Efficient knockdown was achieved after 48h

incubation.

2.3. Mitochondria and cytosol fractionation

Isolation of mitochondria and cytosol fractions was performed as previously described (Wieckowski et al., 2009). Briefly, cells were collected and pelleted by centrifugation at 4°C. Pellets were then resuspended in lysis buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM ethylene glycol tetraacetic acid -EGTA- and 30 mM trisaminomethane hydrochloride pH 7.4) and sonicated for 10 s. Cytosolic lysate was collected after two fractionations by centrifuging at 600 g/5 min and 7000 g/10 min respectively, and stored at -80°C. Remaining pellet was again resuspended in the same lysis buffer without EGTA and centrifuged two times at 7000 g and then 10000 g for 10 min. Pellet containing mitochondria fraction was resuspended in a buffer containing 250 mM mannitol, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) and 0.5-mM EGTA and stored at -20°C.

2.4. Apoptosis rate analysis

Cells at a density of 4 x 10^3 cells /ml were seeded in 96-well-plates, incubated overnight, treated for 24 h with FL3 and collected. Apoptosis rate was then assessed by flow cytometry (Guava EasyCyte Plus, EMD Millipore Corporation, Billerica, MA, USA), using propidium iodide -PI- (Miltenyi Biotec, Paris, France) and annexin V-FITC (Immunotools, Friesoythe, Germany), following the manufacturer's recommendations and as previously described (Ali Azouaou et al., 2015; Sharif et al., 2011).

2.5. Mitochondrial membrane potential (ΔΨm) measurement

Cells seeded at a density of 2 × 10^5 cells/well in 6-well plates, were treated with the vehicle or FL3 during 24 h and harvested. The ΔΨm changes were then determined by incubating the cells for 20 min at 37°C in phosphate-buffered saline, supplemented with 40 nM of [3,3'-dihexyloxacarbocyanine iodide] -DiOC6- (Sigma-Aldrich), prior to flow cytometry examination (Guava EasyCyte Plus).

2.6. Western blot analysis

Proteins from cell lysates were extracted and western blots were performed as described previously (Fuhrmann et al., 1999; Sharif et al., 2013). Target proteins were visualized with the following antibodies: a rabbit polyclonal Akt antibody, a rabbit polyclonal anti-phospho-Akt Ser473 antibody, a mouse monoclonal anti-phospho-Bad Ser112 antibody, a rabbit polyclonal anti-phospho-Bad Ser136 antibody, a rabbit monoclonal phospho-p38 Thr180/Tyr182 MAPK antibody, a rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology), a rabbit polyclonal anti-Bad antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), a mouse monoclonal anti-cytochrome C antibody (BD Biosciences Pharmingen, San Diego, CA, USA), a rabbit polyclonal anti-Cox IV antibody (Thermo Fisher Scientific), a rabbit polyclonal anti-Bax antibody, a rabbit polyclonal anti-Bcl-2 antibody, a mouse polyclonal anti-beta tubulin antibody (Abcam, Paris, France). The chemiluminescent blots were imaged with ImageQuant LAS4000 (GE Healthcare Life Science, Velizy-Villacoublay, France) and band densities were quantified using NIH's Image J software.

2.7. Statistical analysis

All data are presented in a bar graph format and expressed as means ± S.E.M. of at least three independent experiments. Statistical analysis has been achieved with Student's t-test, by using GraphPad Prism software (Prism version 5.04 for Windows, GraphPad Software, La Jolla, CA, USA); p value < 0.05 is considered as significant.

3. Results

3.1. Healthy cells are unresponsive to FL3 cytotoxic activity

In a previous report, we showed that FL3, even at high concentration (2000 nM), selectively inhibits, after 24h, the cell growth of cancer cells (*e.g.* teratocarcinomal NT2/D1 cells), but not of normal cells (*e.g.* fibroblast BJ cells), as measured by MTS or trypan blue assay (Emhemmed et al., 2014). This suggests that FL3 could act as a pro-apoptotic drug which is able to target specifically pathological cells and spare healthy cells. To confirm the selective cytotoxic properties of FL3, several normal or cancer cell lines were exposed to the drug at the same conditions mentioned above and their apoptosis rates were determined by flow cytometric analysis. As shown in Fig. 1A and 1B, 52% and 35 % of NT2/D1 and A375 FL3-treated cells were annexin V-FITC positive respectively, whereas there was no detectable fluorescence for the normal BJ or HaCaT cells. Accordingly, FL3-induced apoptosis in the studied cancer cell lines was associated

with a sharp expression of caspase-3 cleaved subunits (Fig. 1C). Complementary experiments also evidenced that the cancer MCF-7 cell line showed 22 % of apoptotic cells, whereas the normal HGF-1 cell line was unaffected after 24h of treatment with FL3 (data not shown). Taken together, our data are firstly consistent with those we previously obtained with the cell proliferation assay and indicate that the drug can specifically induce a caspase-3 pro-apoptotic process in cancer cells, but not in normal cells. The subsequent experiments were therefore focused on BJ fibroblast and NT2/D1 cell lines.

3.2. Mitochondrial membrane potential ($\Delta\Psi m$) and cytochrome C location are unchanged in FL-3 treated fibroblasts

p38 MAPK activity is known to trigger an intrinsic caspase-3 dependent pro-apoptotic pathway, through the disruption of the mitochondrial membrane and the release of cytochrome C (Zhu et al., 2007). We therefore determined whether FL3 could selectively affect the mitochondrial membrane permeability in both teratocarcinomal and fibroblast cells. For that purpose, we monitored, by flow cytometry, the uptake rate of DiOC6, a mitochondrial specific and voltage-dependent fluorescent dye. As shown in Fig. 2A and 2B, the percentage of NT2/D1 cells that exhibit low levels of DiOC6 was significantly increased when exposed to FL3, reflecting indirectly a loss of $\Delta\Psi m$. At the opposite, no significant change was observed in treated BJ cells, when compared to untreated cells; this demonstrates that their mitochondrial membrane remains intact, while exposed to the drug.

The subcellular location of cytochrome C is an additional parameter which

reflects the integrity of the mitochondria. For that purpose, mitochondrial and cytosolic fractions were collected from treated and untreated teratocarcinomal cells and fibroblasts, and the corresponding expression levels of cytochrome C were analyzed by immunoblotting assays. In a first step, the purity of the fractions was monitored by determining the expression levels of COX IV (cytochrome c oxidase subunit 4) and beta-tubulin, as integral proteins of the mitochondrion or cytosol respectively. Our results showed that cytochrome C expression levels were significantly decreased in the mitochondrial fraction of NT2/D1-treated cells, whereas elevated expression levels were observed in the cytosolic fraction, when compared to untreated cells (Fig. 2C and 2D). Interestingly, cytochrome C expression was restricted to the mitochondria of BJ cells, even after exposure to FL3. Taken together, these observations well support the hypothesis that the inability of the drug to induce a cytotoxic effect in normal cells is mainly due to its failure to alter their mitochondrial membrane.

3.3. FL3 selectively phosphorylates the pro-apoptotic protein Bad in fibroblasts, without any changes on Bax and Bcl-2 expression

It has previously been shown that flavaglines, like rocaglamide or silvestrol, specifically induces in cancer cells, an intrinsic pro-apoptotic process, by targeting the Bcl-2 network, including a mitochondrial docking of Bax and a downregulation of total Bcl-2 (Callahan et al., 2014; Ebada et al., 2011). We hypothesized therefore that the activity of Bcl-2 network could be critical for the fate decision of the cell, either pathological or healthy, to live or die. As expected, we observed a significant increase of Bax expression levels in the mitochondrial fraction of NT2/D1-treated

cells, whereas reduced expression levels were shown in the cytosolic fraction, when compared to untreated cells (Fig. 3A). Moreover, a slight decrease of the expression levels of mitochondrial and cytosolic Bcl-2 was observed in the studied cancer cell line (Fig. 3B). On the contrary, no significant sub-cellular redistribution of both Bax and Bcl-2 could be evidenced in fibroblast-treated cells, when compared to untreated cells (Fig. 3A and 3B). These results suggest, at once, that the cytotoxic drug FL3 is unable to alter, in healthy cells, the activity of the Bcl-2 family members which are specifically targeted in pathological cells and which might have been involved, in an opposite manner, in the resistance of normal cells. The study was therefore extended to another member of this network which has particular features, namely Bad. Indeed, Bad activity, on its own, has a fine-tuned regulation which instructs the action of Bcl-2 network. In that respect, non-phosphorylated Bad can initiate the mitochondrial pro-apoptotic pathway. At the opposite, when phosphorylated, Bad acts as an anti-apoptotic factor (Dai et al., 2016). Therefore, we wondered whether the synthetic flavagline might selectively target Bad in normal cells and prevent the occurrence of the mitochondrial pro-apoptotic process. In order to verify this assumption, total cell lysates of FL3-treated and untreated teratocarcinomal CSLCs and fibroblast NSLCs were subjected to Western blot analysis by using specific antibodies against phospho-Bad at Ser112 or Ser136. Accordingly, FL3-treated BJ cells showed significant amount of phosphorylated Bad at both sites, when compared to untreated cells. In contrast, Bad was clearly unphosphorylated in NT2/D1 treated cells (Fig. 4A and 4B). It should be noted that Bad phosphorylation was also be observed in FL3-treated normal HaCaT cells, but not in FL3-treated A375 cancer cells (Suppl. Fig. S1). Taken together, these results suggest that the activation of Bad in healthy

cells could be the potential molecular event by which they escape from the cytotoxic activity of the synthetic flavagline.

3.4. Transient knockdown of Bad is sufficient to sensitize fibroblast cells to FL3 cytotoxicity

To verify the protective effect of Bad phosphorylation against the cytotoxic activity of FL3 on fibroblasts, we next targeted Bad by silencing its expression with specific siRNA. Prior to the treatment, a significant transient knockdown of the protein has been observed in transfected cells, when compared to non-transfected cells. Upon treatment with FL3 for 48h, transfected normal cells significantly responded to the toxic effect of FL3 and underwent apoptosis, as measured by annexin V-FITC assay (Fig. 5A and 5B). Accordingly, western blot analysis confirmed this response since increasing levels of cleaved caspase- 3 could only be detected in transfected cells, concomitantly with a strong decrease of the expression levels of Bad and its phosphorylated forms (Fig. 5C and 5D). Collectively, these results strongly suggest that phosphorylation of Bad is an important molecular event implicated in the protection of fibroblasts against FL3 cytotoxicity.

3.5. FL3 markedly phosphorylates Akt in fibroblast cells

It has been reported that the activation of the upstream regulator of Bad, namely Akt, plays a central role in inhibiting the mitochondrial pro-apoptotic pathway (Matheny and Adamo, 2009; Mitsiades et al., 2004). We therefore

examined by Western blot analysis whether FL3 could selectively activate in fibroblasts Akt, through its phosphorylation at Ser473. The results showed that FL3 treatment induced after 48h the phosphorylation of the pro-survival factor in the normal cells, although the expression levels of total Akt remained unchanged (Fig. 6A and 6B). It should be noticed that no significant changes in the expression levels of both total and phosphorylated Akt could be observed in NT2/D1 teratocarcinomal cells (data not shown). To explore the involvement of Ser473 phosphorylated form of Akt in FL3-related survival process on normal cells, we used LY294002, an established inhibitor of Akt activity. As shown in Fig. 6C and 6D, the presence of this inhibitor sensitized fibroblasts to FL3 which underwent a pronounced apoptosis after 48h, as measured by annexin V-FITC assays. These data emphasize therefore the implication of Akt phosphorylation in the cytoprotective mechanism adopted by the fibroblasts against FL3 cytotoxicity.

4. Discussion

The molecular mechanisms by which flavagline derivatives exert their anticancer activity against various cancer cell models are well documented (Basmadjian et al., 2013; Ribeiro et al., 2012). However, the mechanisms that ensure the protection of normal cells against their cytotoxic activity remain to be clarified. Accordingly, we previously showed that the synthetic flavagline FL3 selectively induces a caspase-3-dependent pro-apoptotic process in teratocarcinoma cells (Emhemmed et al., 2014), whereas it has no obvious effect on fibroblasts. The aim of the present work was therefore to investigate the underlying molecular process implicated in the absence of damaging effects of FL3

towards the normal cells, including fibroblastic cells.

The study firstly provides further evidences for the selective cytotoxic activity of FL3 on cancer cells *versus* normal cells. As expected, annexin V-FITC assays, as well as the determination of the expression levels of activated caspase-3, show that the different cancer cell lines studied respond with a more or less sensitivity to the drug, irrespective of their cell type of origin and their degree of malignancy. At the opposite, FL3 is unable to induce apoptosis in the different treated healthy cell lines, even those which share a neighbor tissular phenotype with the cancer cell lines studied (ie HaCaT keratinocytes versus A375 melanoma cells). This signifies that the selective cytotoxic effect of the drug is associated with a pathological background and that the tissular phenotypic variations have little incidence on the biological activity of FL3.

The present study provides novel insights into the molecular mechanisms that prevent the cytotoxic activity of FL3 on normal cells. As mentioned above, one of the putative targets of the synthetic flavagline could be Bad which behaves like a two-faced “Janus”, acting either as a pro-apoptotic or antiapoptotic factor and thereby modulating the activity of Bcl-2 network. Accordingly, FL3 treatment induces a strong phosphorylation of Bad in fibroblasts and keratinocytes, but not in teratocarcinomal and melanoma cells. Such phosphorylation is known to allow Bad to be sequestered in the cytosol by binding to the cytosolic protein 14-3-3 and therefore prevents the displacement of Bax from Bcl-2, which in turn impedes apoptosis (Datta et al., 1997; Masters et al., 2001). The protective role of Bad phosphorylation against FL3 cytotoxicity on fibroblasts has been further highlighted

by knock-down experiments. Indeed, we observed that the loss of Bad function with siRNA sensitizes fibroblastic cells to the pro-apoptotic activity of FL3. This strongly suggests that Bad phosphorylation is the central molecular event for the protection of normal cells against the cytotoxic effects of flavagline derivatives.

It has been proven that Akt activity prevents mitochondrial apoptosis by blocking the pro-apoptotic function of Bad through its phosphorylation (Datta et al., 1997); it could therefore be hypothesized that Akt activation may serve as an upstream inducer of Bad phosphorylation in FL3-treated fibroblasts. In this regard, we observe that the drug is able to trigger Akt phosphorylation at Ser473 in fibroblasts, whereas total amount of Akt remains unchanged. Moreover, by inhibiting Akt activity with the specific inhibitor LY294002, a sensitization of fibroblasts to the cytotoxic effect of FL3 occurs, suggesting an essential role of Akt activation in the resistance of fibroblasts to the drug. It should be noted that LY294002 inhibits FL3-induced Akt phosphorylation 24h after the treatment (data not shown), whereas evidence of a cytotoxic response is only detected after 48 h of treatment. These results are consistent with previous observations, which showed that LY294002 rapidly, but transitionally, abolishes Akt phosphorylation, leading to a delay of apoptosis which remains temporary. This event is likely linked to the short half-life of LY294002 which does not allow sustained inhibition of Akt activity (Werzoma et al., 2009). Taken as a whole, our results suggest that FL3 induces an activation of Bad in fibroblasts via the phosphorylation of Akt. It should be noticed that expression levels of total Akt are stable and unchanged in both normal and cancer cells, after FL3 treatment. Therefore, how the drug selectively phosphorylates Akt in non-cancerous cells, will need to be clarified in further

studies.

Interestingly, we previously observed that the pharmacological agent triggers apoptosis in teratocarcinomal cells, through an activation of p38 MAPK (Emhemmed et al., 2014). In agreement with these results, another study revealed that the activation of this regulator by rocaglamide is one of the earliest events which is responsible for the induction of apoptosis in leukemia cells (Zhu et al., 2007). Moreover, it has also been reported that rocaglamide can increase the phosphorylation levels of p38 MAPK in normal T cells (Proksch et al., 2005). Accordingly, the current study shows that FL3 is also able to induce a pronounced phosphorylation of p38 MAPK in fibroblast cells (Suppl. Fig. 2). These different works suggest therefore that the drug is, at once, able to activate p38 MAPK in any cell type. The preferential induction by flavaglines of a pro-survival process in normal, but not in tumor cells, is therefore likely associated with a differential activation of several specific stress-responses, including a mandatory activation of p38 MAPK and defined rescue targets. In the current study, we observe that both Bad and p38 MAPK are phosphorylated in FL3-treated fibroblasts; our results suggest firstly an absence of a direct effect of p38 MAPK on Bad activity, thereby on cell survival. Moreover, forced inhibition of FL3-induced activation of Bad initiates a pro-apoptotic process in fibroblasts. We hypothesize therefore that the drug targets different signaling pathways and that the resulting effect, in particular on Bcl-2 interactome, allows skin cells to escape from the p38 MAPK-induced pro-apoptotic process (see graphical abstract).

In conclusion, the synthetic flavagline FL3 selectively kills cancer cells, while

sparing normal cells from its cytotoxic activity. Moreover, the drug actively induces a pro-survival mechanism in healthy skin cells, by targeting an elective nodal point of the Bcl-2 network, namely Bad. It should be noted that only few classes of pharmacological agents are able to induce an anti-proliferative process on cancer cells and, adversely, a pro-survival process in normal cells. For instance, polyphenols, like sulforaphane or resveratrol, have these bivalent properties (Briones-Herrera et al., 2018; Rauf et al., 2017), in relation with their potential as cellular stress initiators, to trigger opposite responses, depending on the cellular background, either normal or pathological. In this point of view, it has been reported that FL3 has, besides its cytotoxic activity on cancer cells, a protective effect on normal cells, like cardiomyocytes (Qureshi et al., 2015) or neural cells (Fahrig et al.,2005). As a consequence, it can be assumed that FL3 could act as a potent anticancer compound with cytoprotective properties on noncancerous cells and therefore should attract much more attention in the future for therapeutic purposes.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1. Effects of FL3 on the apoptosis rate of different healthy and cancer cells. Cells were treated with FL3 at the indicated concentration for 24h. (**A**) Scatter plots show percentage of living (lower left panel), early apoptotic (lower right panel), late apoptotic (upper right panel) and necrotic (upper left panel) cells, measured by flow cytometry, using annexin V-FITC/PI staining assay. (**B**) Bar charts represent the percentage of annexin V-positive cells. The number of apoptotic bodies is expressed as percent relative to the total cell number. (**C**) Total cell lysates were subjected to immunoblotting analysis with the corresponding antibodies. Specific bands were detected with their expected apparent molecular weight. The panels show representative immunoblotting results. Values are mean ± S.E.M of at least three independent experiments; statistically significant: **, $p < 0,01$; ***, $p < 0,001$.

Fig. 2. Effect of FL3 on mitochondrial integrity of fibroblasts and teratocarcinomal cells. Cells were treated with FL3 at the indicated concentration for 24h. (**A**) Histograms show the percentage of cells exhibiting $\Delta\Psi$m changes, as measured by flow cytometry, using DiOC6 staining assay. Fluorescence intensity shifts from right to left indicate a loss of $\Delta\Psi$m. (**B**) Bar charts represent the percentage of cells with mitochondrial permeability alteration, expressed as percent relative to the total number of cells. (**C**) Expression levels of cytochrome C, COX IV and β-tubulin were assessed in BJ or NT2/D1 cells by western blot analysis. Specific bands were detected with their expected apparent molecular weight. The panel shows representative immunoblotting results. COX IV and β-

tubulin were used as mitochondrial and cytosolic internal controls respectively. (**D**) Bar charts show densitometry results of mitochondrial and cytosolic cytochrome C expression levels, normalized to either COX IV or β-tubulin expression levels. Values are mean ± S.E.M of three independent experiments; statistically significant: **, $p < 0,01$; ****, $p < 0,0001$.

Fig. 3. Effect of FL3 on mitochondrial and cytosolic distribution of Bax and Bcl-2 in fibroblasts and teratocarcinomal cells. Cells were exposed to the indicated concentration of FL3 for 24h. Mitochondrial and cytosolic fractions were subjected to immunoblotting analysis by using antibodies against Bax (**A**) or Bcl-2 (**B**). Specific bands were detected with their expected apparent molecular weight. The panels show representative immunoblotting results. Bar charts show densitometry results for the indicated proteins, normalized to either COX IV or β-tubulin expression levels. Values are means ± S.E.M. of at least three independent experiments; statistically significant: *, $p < 0.05$.

Fig. 4. Effect of FL3 on Bad phosphorylation in fibroblasts and teratocarcinomal cells. Cells were exposed to the indicated concentration of FL3 for 24h. (**A**) Total cell lysates were subjected to immunoblotting analysis by using antibodies against total Bad, p-Bad Ser112 or p-Bad Ser136. Specific bands were detected with their expected apparent molecular weight. The panels show representative immunoblotting results. (**B**) Bar charts show densitometry results for the indicated proteins, normalized to total Bad expression levels. Values are means ± S.E.M. of at least three independent experiments; statistically significant: **, $p < 0.01$.

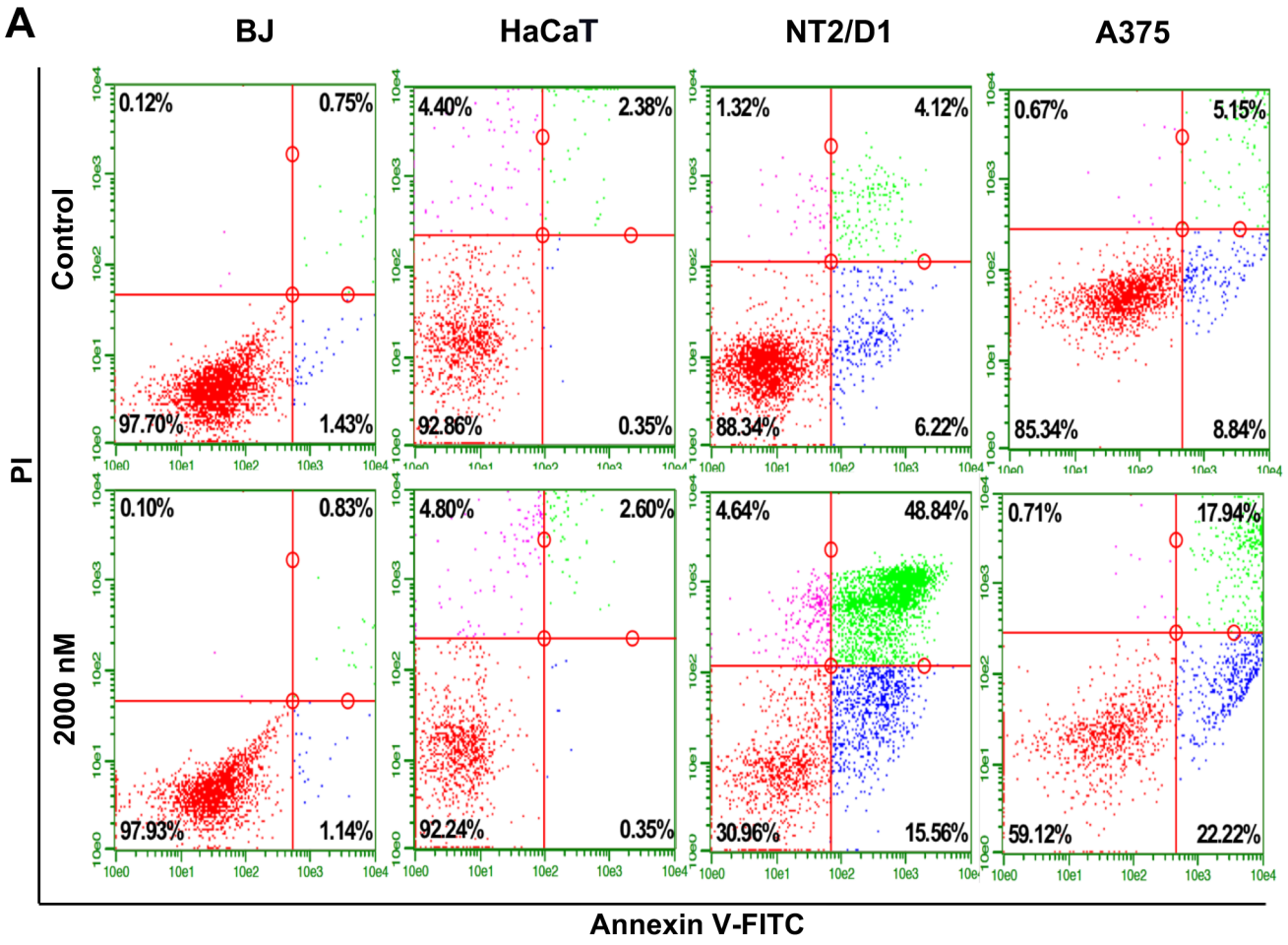
Fig. 5. Abrogation in fibroblasts of the cytoprotection against FL3 by posttranscriptional silencing of Bad. Cells were transfected with 120 nM of Bad siRNA for 48h, followed by an additional 48h incubation in the presence or absence of 2000 nM FL3. (**A**) Scatter plots show the percentage of cells in different stages, as mentioned in Fig. 1A. (**B**) Bar graphs represent the number of apoptotic cells, calculated as the sum of both early and late apoptotic bodies, and expressed as percent relative to the total cell number. (**C**) Panels show representative immunoblotting results for total Bad, p-Bad Ser112, p-Bad Ser136 or cleaved caspase-3. Specific bands were detected with their expected apparent molecular weight. (**D**) Bar charts show densitometry results for the indicated proteins, after normalization against β-tubulin expression levels and given as ratios relative to the value obtained for the control sample. Values are means ± S.E.M. of three independent experiments; statistically significant: **, $p < 0.01$ (*versus* untreated group).

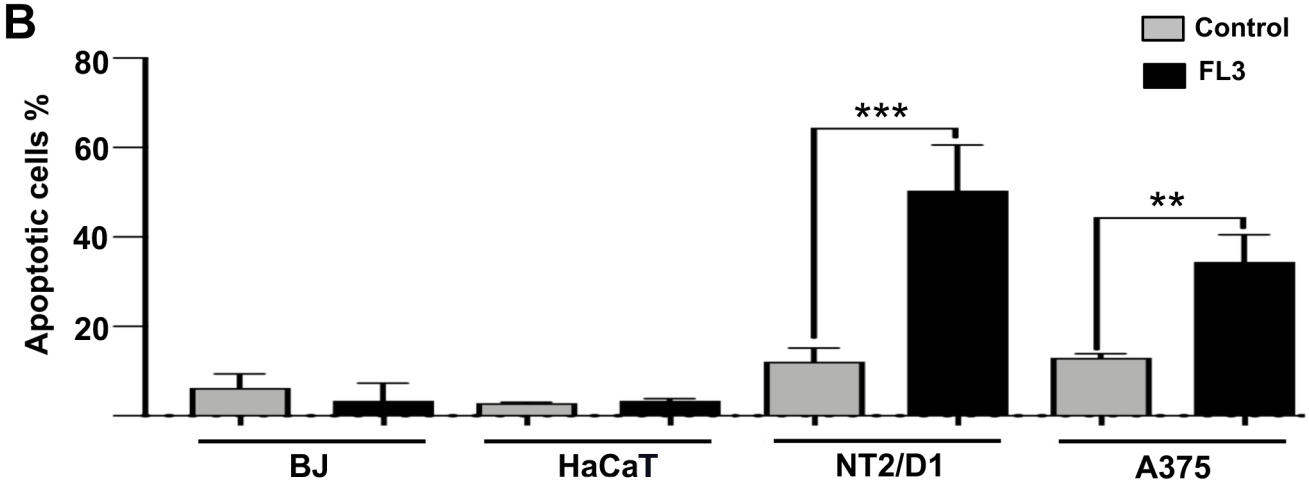
Fig. 6. Implication of Akt activation in the selective cytoprotection of fibroblasts against FL3. (**A**) Total cell lysates of untreated or 24h FL3-treated cells were subjected to immunoblotting analysis by using antibodies against total Akt or p-Akt Ser473. The panels show representative immunoblotting results. (**B**) Bar graphs show quantitative densitometric analysis of p-Akt expression levels, normalized to the corresponding total Akt expression levels. (**C**) Fibroblasts were pretreated for 1h with 40 μM of LY249002, followed by an additional 48h incubation, in the presence or absence of 2000 nM FL3. Scatter plots show the percentage of cells in different stages, as mentioned in Fig.1A. (**D**) Bar graphs

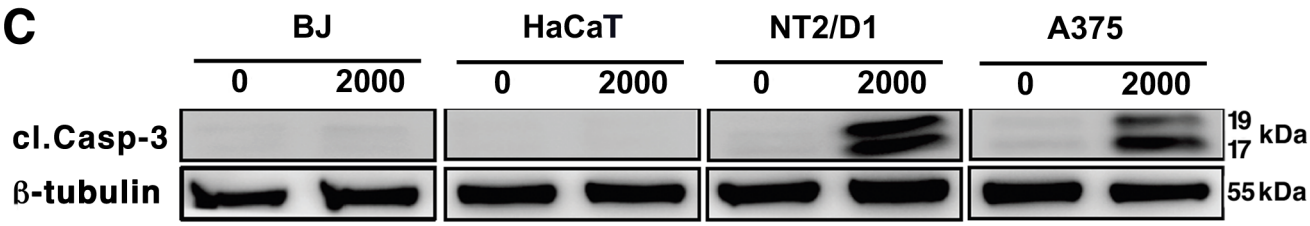
represent the number of apoptotic fibroblasts incubated at the indicated conditions, calculated as the sum of both early and late apoptotic bodies and expressed as percent relative to the total cell number. Values are means ± S.E.M. of three independent experiments; statistically significant: **, $p < 0.01$; ****, $p < 0.0001$.

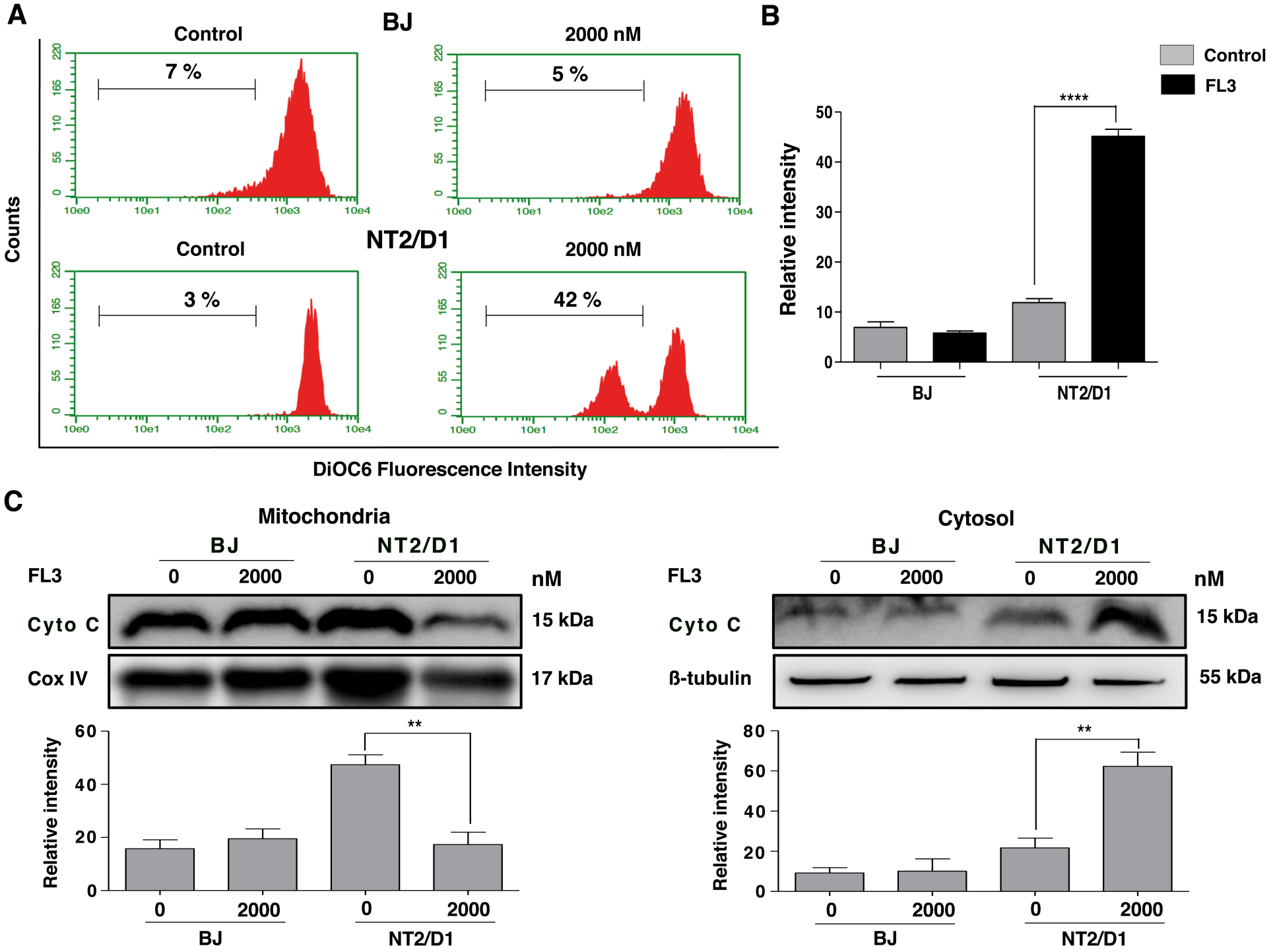
Suppl. Fig. 1. Effects of FL3 on Bad phosphorylation in HaCat normal cells (A) or A375 cancer cells (B). Cells were exposed to the indicated concentration of FL3 for 24h. Total cell lysates were subjected to immunoblotting analysis by using antibodies against total Bad or p-Bad Ser136. Specific bands were detected with their expected apparent molecular weight. The panels show representative immunoblotting results.

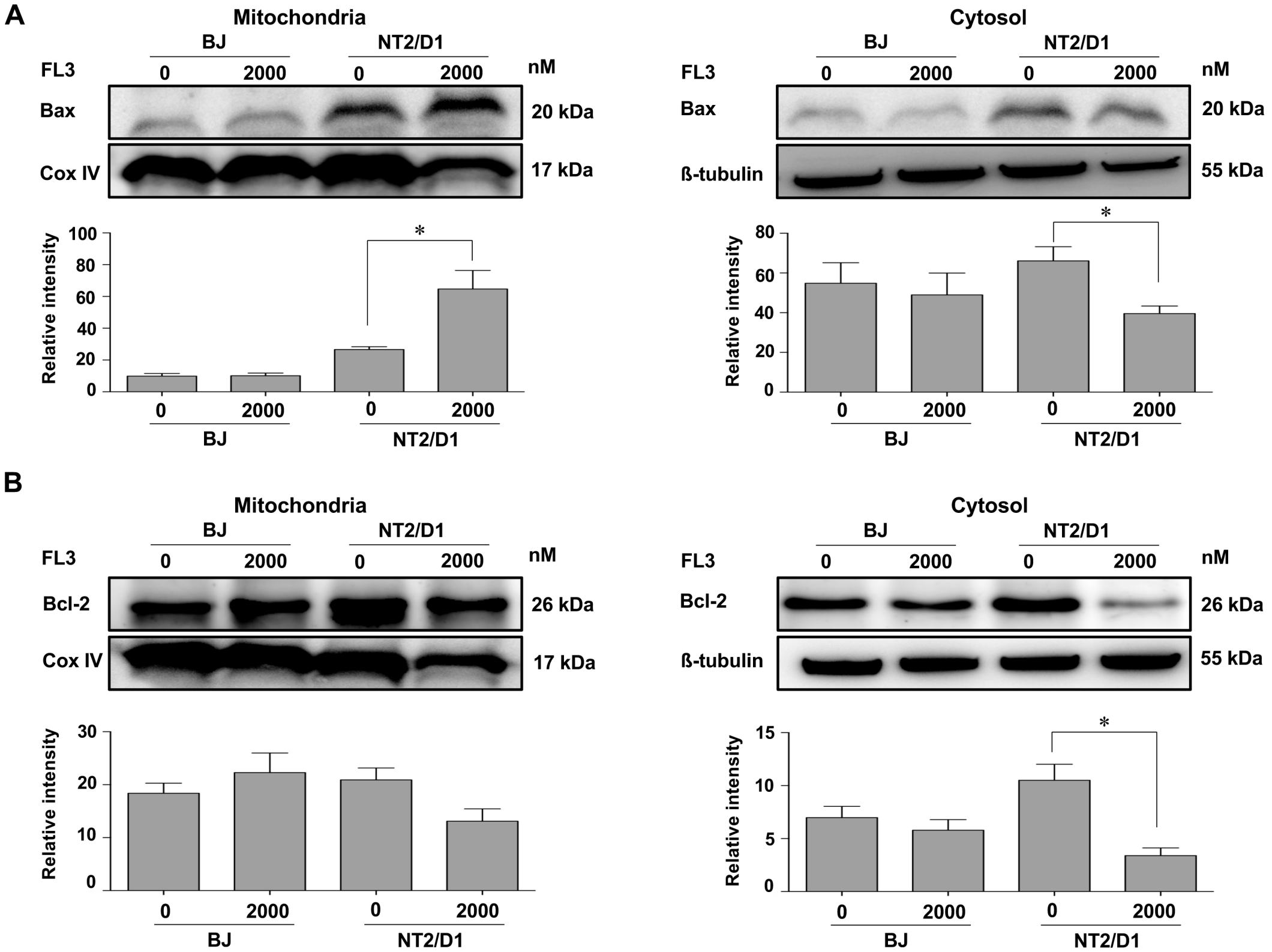
Suppl. Fig. 2. Effects of FL3 on p38 MAPK and cleaved caspase-3 activities of fibroblasts and teratocarcinomal cells. Cells were treated with FL3 at the indicated concentration for 24h. (**A**) Total cell lysates were subjected to immunoblotting analysis with the corresponding antibodies. Specific bands were detected with their expected apparent molecular weight. The panels show representative immunoblotting results. (**B**) Bar charts show densitometry results of p38 MAPK expression levels normalized to β-tubulin expression levels. Values are mean ± S.E.M of at least three independent experiments; statistically significant: *, $p < 0,05$; **, $p < 0,01$.

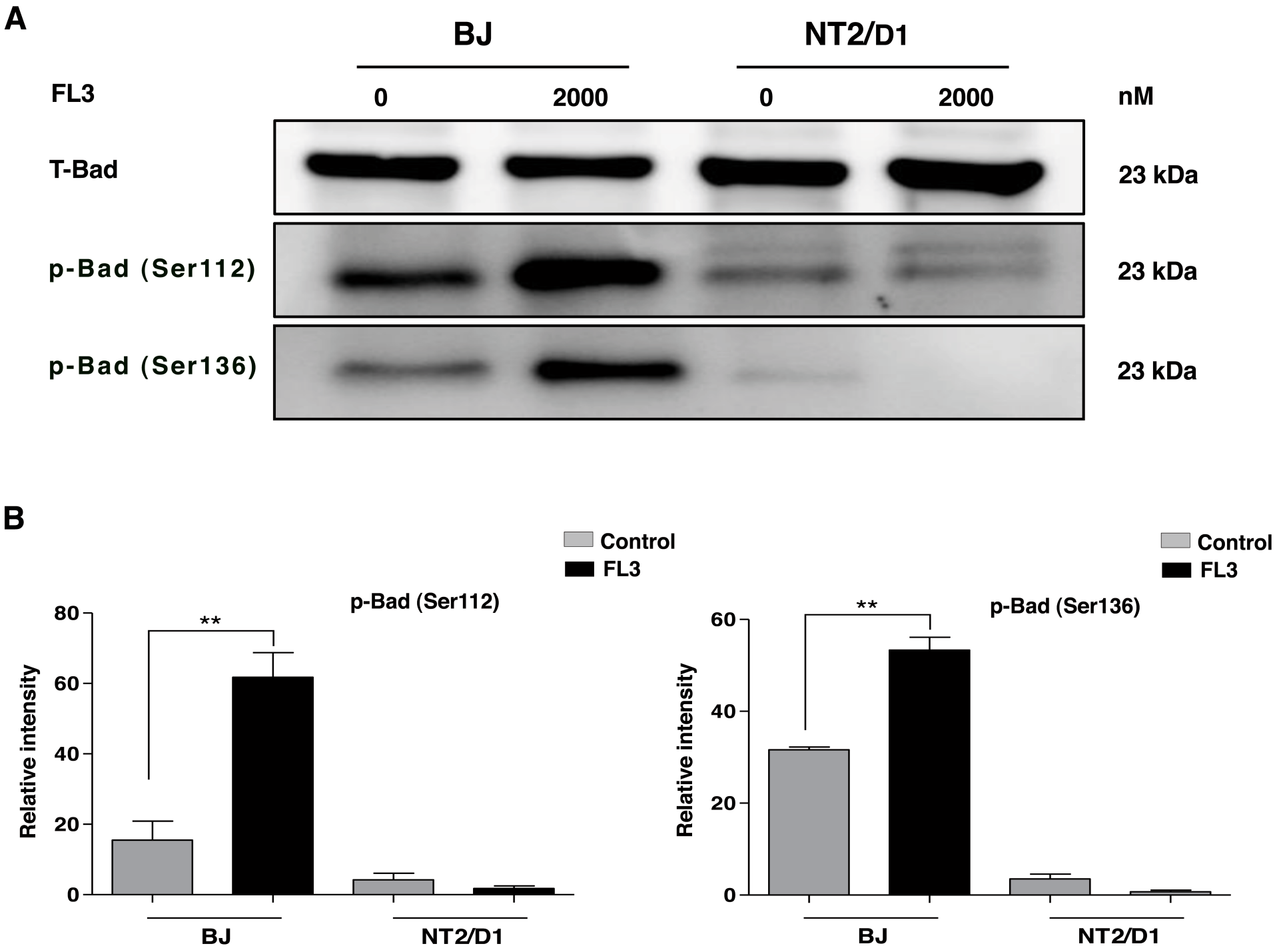

A
BJ
HaCaT
NT2/D1
A375
Control
2000 nM
PI
Annexin V-FITC
0.12%
0.75%
97.70%
1.43%
4.40%
2.38%
92.86%
0.35%
1.32%
4.12%
88.34%
6.22%
0.67%
5.15%
85.34%
8.84%
0.10%
0.83%
97.93%
1.14%
4.80%
2.60%
92.24%
0.35%
4.64%
48.84%
30.96%
15.56%
0.71%
17.94%
59.12%
22.22%

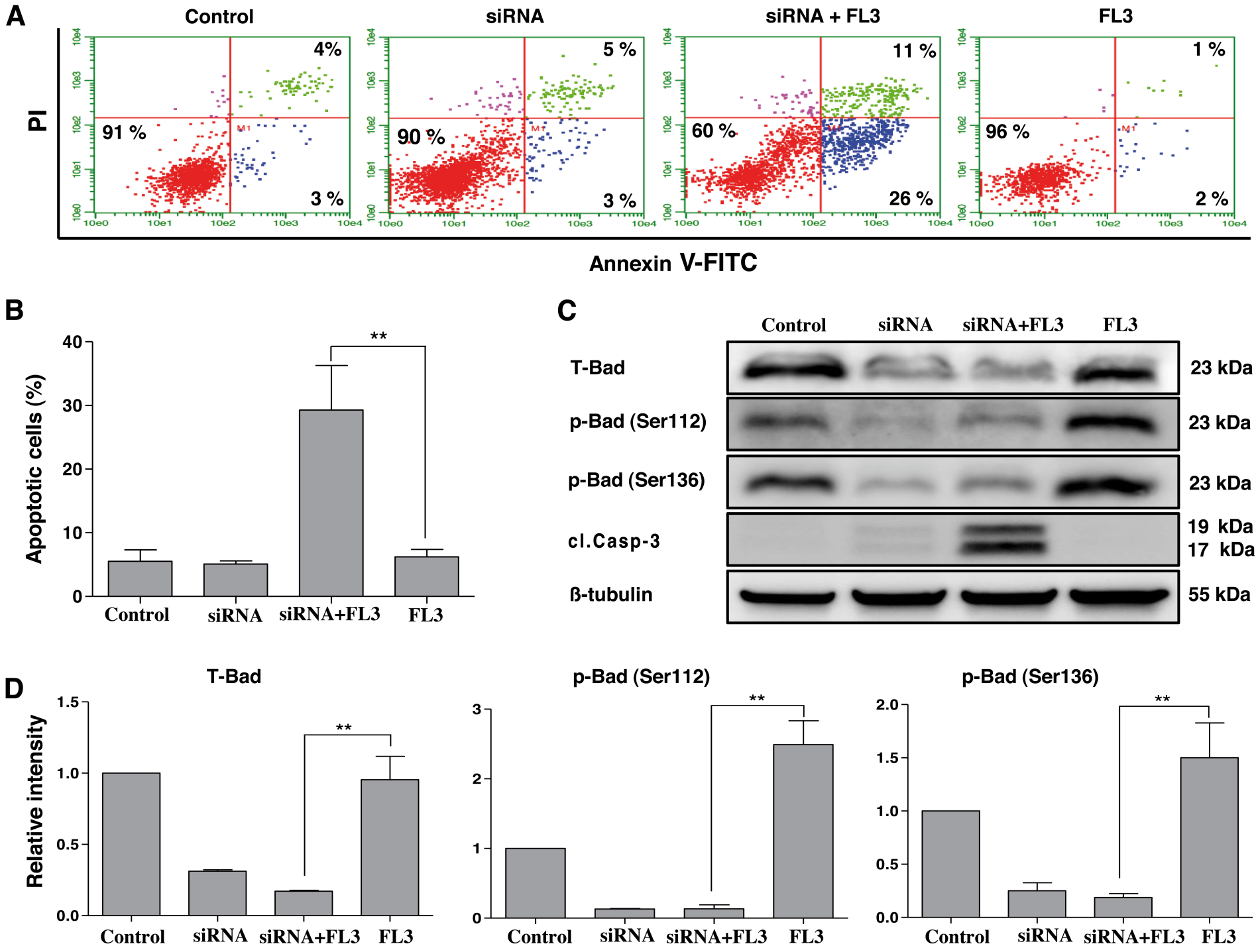

B
Control
FL3
Apoptotic cells %
80
60
40
20
***
**
BJ
HaCaT
NT2/D1
A375

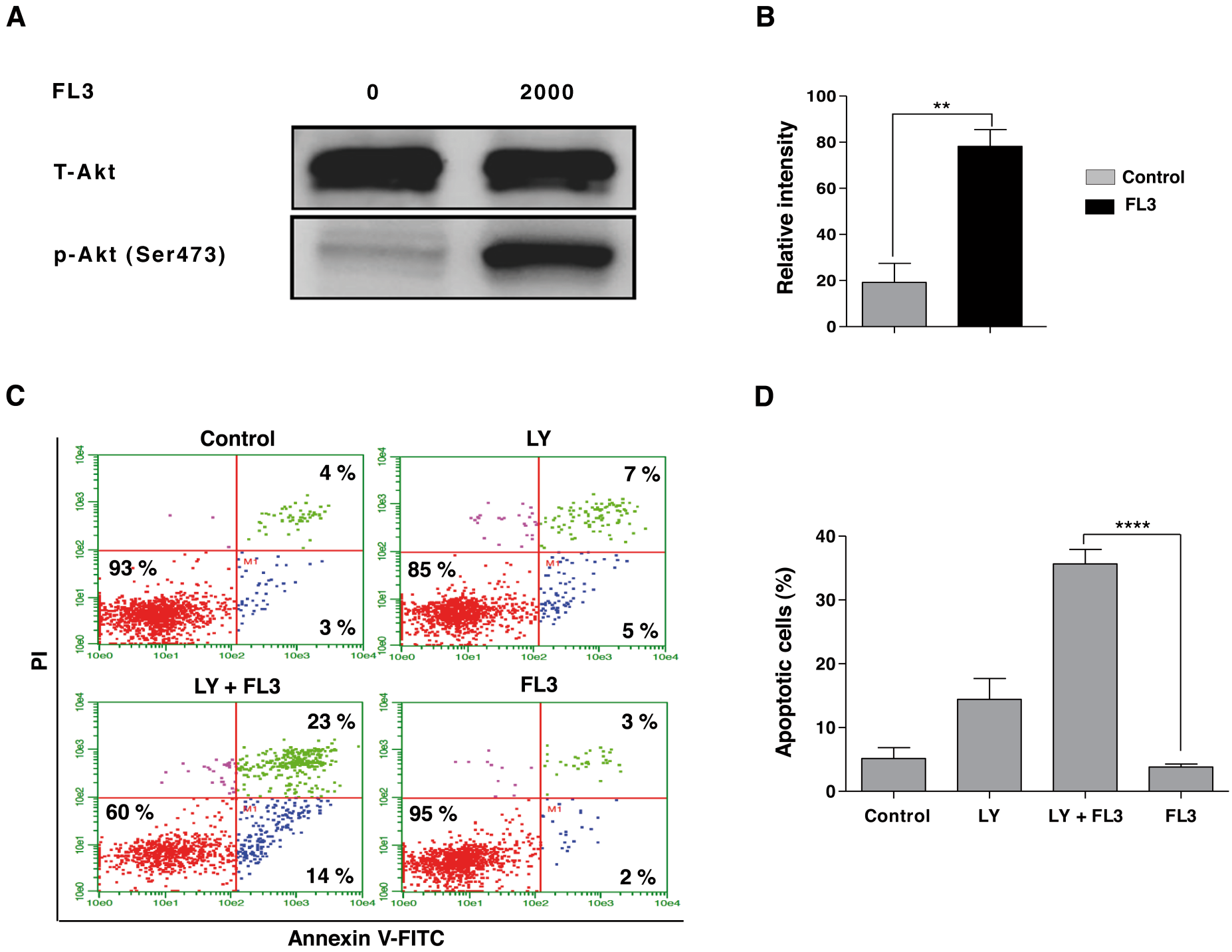

C
BJ
HaCaT
NT2/D1
A375
0
2000
cl.Casp-3
β-tubulin
19
17
kDa
55 kDa


A
BJ
Control
7 %
2000 nM
5 %
NT2/D1
Control
3 %
2000 nM
42 %
Counts
DiOC6 Fluorescence Intensity
B
Control
FL3
****
Relative intensity
BJ
NT2/D1
C
Mitochondria
BJ
NT2/D1
FL3
0
2000
0
2000
nM
Cyto C
15 kDa
Cox IV
17 kDa
**
Relative intensity
BJ
NT2/D1
Cytosol
BJ
NT2/D1
FL3
0
2000
0
2000
nM
Cyto C
15 kDa
ß-tubulin
55 kDa
**
Relative intensity
BJ
NT2/D1


A
Mitochondria
BJ
NT2/D1
FL3
0
2000
0
2000
nM
Bax
20 kDa
Cox IV
17 kDa
Relative intensity
100
80
60
40
20
0
*
0
2000
0
2000
BJ
NT2/D1
Cytosol
BJ
NT2/D1
FL3
0
2000
0
2000
nM
Bax
20 kDa
ß-tubulin
55 kDa
Relative intensity
80
60
40
20
0
*
0
2000
0
2000
BJ
NT2/D1
B
Mitochondria
BJ
NT2/D1
FL3
0
2000
0
2000
nM
Bcl-2
26 kDa
Cox IV
17 kDa
Relative intensity
30
20
10
0
0
2000
0
2000
BJ
NT2/D1
Cytosol
BJ
NT2/D1
FL3
0
2000
0
2000
nM
Bcl-2
26 kDa
ß-tubulin
55 kDa
Relative intensity
15
10
5
0
*
0
2000
0
2000
BJ
NT2/D1

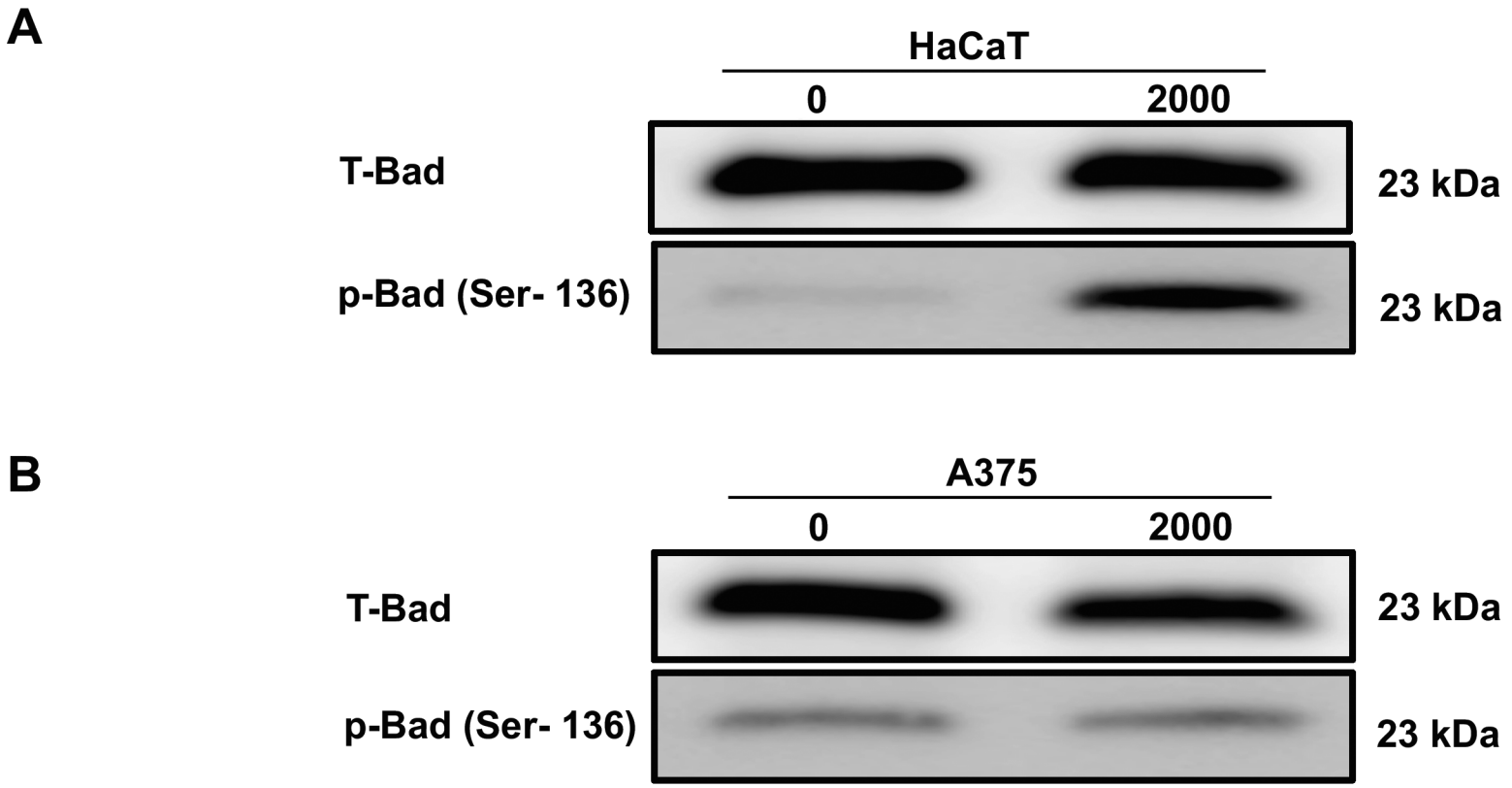

A
BJ
NT2/D1
FL3
0
2000
0
2000
nM
T-Bad
23 kDa
p-Bad (Ser112)
23 kDa
p-Bad (Ser136)
23 kDa
B
Control
FL3
p-Bad (Ser112)
Relative intensity
80
60
40
20
0
**
BJ
NT2/D1
Control
FL3
p-Bad (Ser136)
Relative intensity
60
40
20
0
**
BJ
NT2/D1


A
Control
siRNA
siRNA + FL3
FL3
PI
Annexin V-FITC
4%
91 %
3 %
5 %
90 %
3 %
11 %
60 %
26 %
1 %
96 %
2 %
B
Apoptotic cells (%)
Control
siRNA
siRNA+FL3
FL3
**
C
Control
siRNA
siRNA+FL3
FL3
T-Bad
p-Bad (Ser112)
p-Bad (Ser136)
cl.Casp-3
ß-tubulin
23 kDa
23 kDa
23 kDa
19 kDa
17 kDa
55 kDa
D
T-Bad
p-Bad (Ser112)
p-Bad (Ser136)
Relative intensity
**


A
FL3
0
2000
T-Akt
p-Akt (Ser473)
B
Relative intensity
100
80
60
40
20
0
**
Control
FL3
C
Control
LY
LY + FL3
FL3
4 %
93 %
3 %
7 %
85 %
5 %
23 %
60 %
14 %
3 %
95 %
2 %
M1
PI
Annexin V-FITC
10e0
10e1
10e2
10e3
10e4
D
Apoptotic cells (%)
40
30
20
10
0
****
Control
LY
LY + FL3
FL3

Conflict of interest

The authors declare no conflict of interest.


A
HaCaT
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23 kDa
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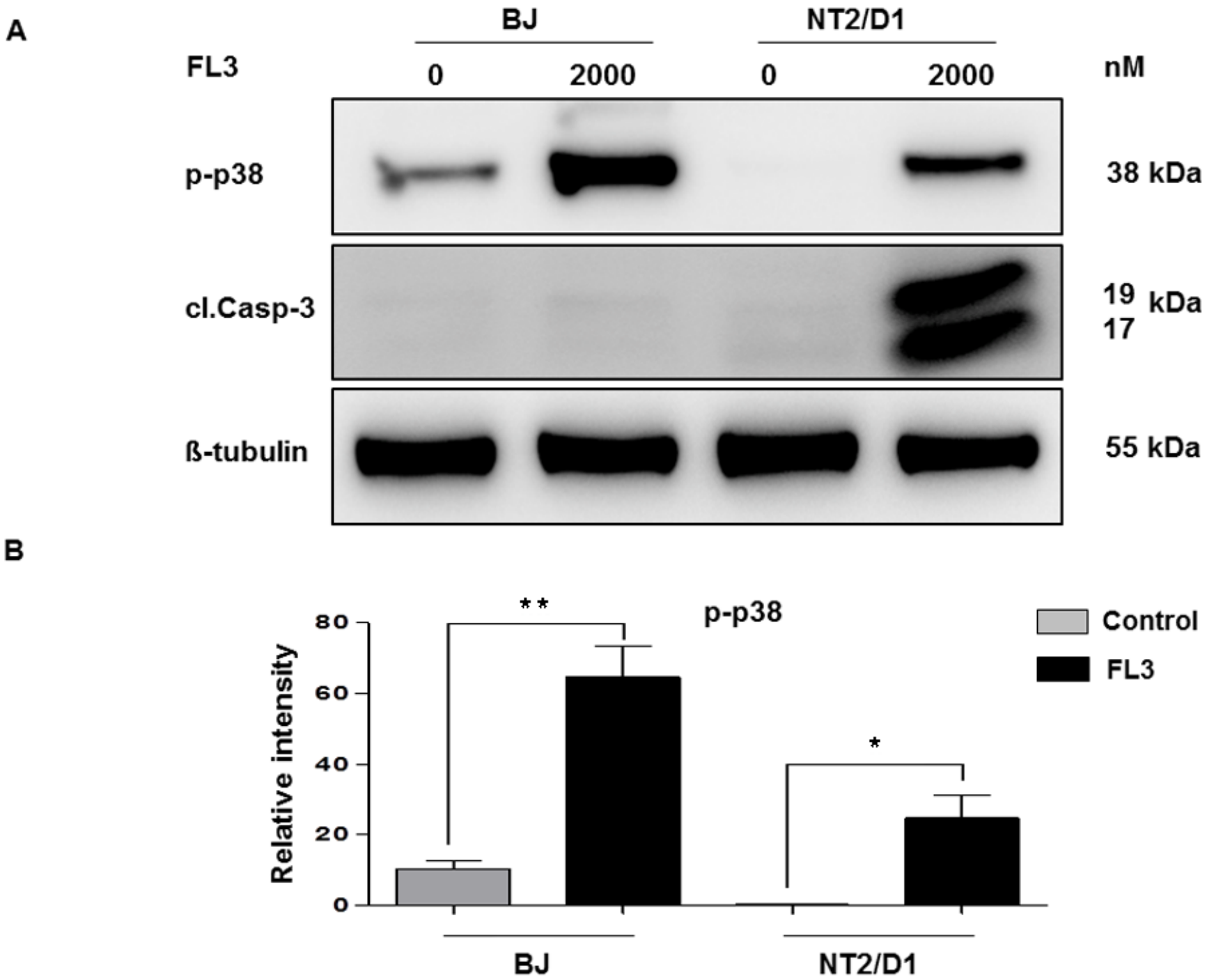
p-Bad (Ser- 136)
23 kDa


A
BJ
NT2/D1
FL3
0
2000
0
2000
nM
p-p38
38 kDa
cl.Casp-3
19
17
kDa
ß-tubulin
55 kDa
B
p-p38
**
*
Control
FL3
Relative intensity
80
60
40
20
0
BJ
NT2/D1