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# Rapamycin inhibits doxorubicin-induced NF-κB/Rel nuclear activity and enhances the apoptosis of melanoma cells☆

Maria Fiammetta Romano [a,*], Raffaella Avellino [a], Antonello Petrella [b], Rita Bisogni [a], Simona Romano [a], Salvatore Venuta [c]

[a] *Department of Biochemistry and Medical Biotechnologies, Federico II University, Via S. Pansini, 5, 80131 Naples, Italy*
[b] *DIFARMA, University of Fisciano, Via Ponte don Melillo, 84084 Fisciano, Salerno, Italy*
[c] *Department of Clinical and Experimental Medicine, Magna Graecia University, 88100 Catanzaro, Italy*

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**Abstract**

Inhibition of nuclear factor (NF)-κB/Rel can sensitise many tumour cells to death-inducing stimuli, including chemotherapeutic agents, and there are data suggesting that disruption of NF-κB may be of therapeutic interest in melanoma. We found that rapamycin sensitised a human melanoma cell line, established from a patient, to the cytolytic effects of doxorubicin. Doxorubicin is a striking NF-κB/Rel-inducer, we therefore investigated if rapamycin interfered with the pathway of NF-κB/Rel activation, i.e. IκBα-phosphorylation, -degradation and NF-κB/Rel nuclear translocation, and found that the macrolide agent caused a block of IKK kinase activity that was responsible for a reduced nuclear translocation of transcription factors. Western blots for Bcl-2 and c-IAP1 showed increased levels of these anti-apoptotic proteins in cells incubated with doxorubicin, in accordance with NF-κB/Rel activation, while rapamycin clearly downmodulated these proteins, in line with its pro-apoptotic ability. The effect of the macrolide on NF-κ B/Rel induction appeared to be independent of the block in the PI3k/Akt pathway, because it could not be reproduced by the phosphatidyl inositol 3 kinase (PI3k) inhibitor, wortmannin. Recently, the immunophilin, FKBP51, has been shown to be essential for the function of IKK kinase. We found high expression levels of FKBP51 in melanoma cells. Moreover, we confirmed the involvement of this immunophilin in the control of IKK activity. Indeed, IκBα could not be degraded when FKBP51 was downmodulated by short-interfering RNAs (siRNAs). These findings provide a possible mechanism for the downmodulation of NF-κB by rapamycin, since the macrolide agent specifically inhibits FKBP51 isomerase activity. In conclusion, our study demonstrates that rapamycin blocked NF-κB/Rel activation independently of PI3k/Akt inhibition suggesting that the macrolide agent could synergise with NF-κB-inducing anti-cancer drugs in PTEN-positive tumours.
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*Keywords:* NF-κB/Rel; Apoptosis; Rapamycin; Melanoma

## 1. Introduction

Rapamycin, a conventional immunosuppressant agent that is used to prevent immunological rejection in organ transplantations, has recently been shown to have anti-cancer effects, decreasing cell proliferation and increasing apoptosis [1–3]. Its anti-cancer activity is classically ascribed to its binding to FKBP12 [4] and forming a complex that inhibits the serine/threonine kinase – mammalian target of rapamycin (mTOR) [5–7]. mTOR, in response to growth factors, hormones, [8,9] mitogens and amino acids [9–11] is activated through the phosphatidyl inositol 3 kinase (PI3k) (PI3k)/Akt cascade [8,9] and regulates protein translation, cell cycle progression and cellular proliferation

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* Corresponding author. Tel.: +39 81 7463125; fax: +39 81 7463653.
*E-mail address:* romano@dbbm.unina.it (M.F. Romano).

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[9–11]. Rapamycin was found to inhibit the oncogenic transformation of human cells induced by either PI3k or Akt [12]. Thus, it is expected to act as an anti-cancer agent, in neoplasias that lack the tumour suppressor gene, PTEN [13]. This gene encodes a lipid and protein phosphatase that, by reducing the cellular levels of phosphatidylinositol triphosphate, antagonises the action of PI3k [14–17]. Nevertheless, data suggests rapamycin inhibits the induction of NF-κB/Rel after different stimuli in various cell types [18–20]. In addition, these findings have recently been strongly supported by the discovery that the rapamycin-binding immunophilin, FKBP51, is an important cofactor of the IKK-α subunit of the IκB-kinase (IKK) complex [21]. NF-κB transcriptional activity is normally inhibited by IκB proteins that sequester it in the cytoplasm [22]. The 700–900 kD IKK complex phosphorylates two critical serine residues (S32 and S36) in IκBs, triggering events that lead to the proteolytic degradation of these inhibitors [23,24], and thereby allowing nuclear translocation of NF-κB/Rel proteins. Active NF-κB factors modulate the expression of a number of genes that sustain cell survival [25–28] and it is widely described that inhibition of this transcriptional activity sensitises many tumour cells to death-inducing stimuli, including chemotherapeutic agents [26,29,30]. Data suggesting that rapamycin was able to inhibit the NF-κB/Rel nuclear activity induced by CD28 in Jurkat cells [18], by insulin in myoblasts [19] and by lipopolysaccharide (LPS) in rat hepatocytes [20], prompted us to investigate if this macrolide agent might, also inhibit chemotherapy induced-NF-κB and overcome drug resistance in aggressive tumours, such as melanoma. Indeed, melanomas are known to be poorly responsive to current anti-cancer drugs, but disruption of NF-κB has been shown to be of therapeutic utility [30]. To this end, we utilised the anthracycline drug, doxorubicin, that activates NF-κB/Rel transcription factors [31] and studied whether rapamycin was able to inhibit the induction of NF-κB/Rel and promote apoptosis in a human melanoma cell line established from a patient.

## 2. Materials and methods

### 2.1. Cells and reagents

A melanoma cell line was established from a patient's primary tumour and was provided by Dr. Gabriella Zupi (Experimental Preclinic Laboratory, Regina Elena Institute for Cancer Research, Roma, Italy). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (ICN Biomedicals, Ohio, USA) supplemented with 10% heat-inactivated foetal calf serum (FCS) (v/v), glutamine and antibiotics at 37 °C in a 5% $CO_2$ humified atmosphere. Purified primary T lymphocytes were obtained from heparinised fresh peripheral blood of healthy donors by a two step-centrifugation, first through a Ficoll–Hipaque (ICN Biomedicals, Ohio, USA) and second 50% Percoll (v/v) (ICN Biomedicals, Ohio, USA) density gradient. The purified cells were >93% $CD3^+$. Rapamycin (Rapamune, Wyeth Ayerst Laboratories. Marietta, PA), doxorubicin hydrocloride (Sigma–Aldrich. St. Louis, Missouri) and wortmannin (Sigma–Aldrich) were used at the concentrations indicated. Cells were preincubated for 20 h with rapamycin or wortmannin before adding doxorubicin.

### 2.2. Analysis of cell death

For analysis of cell viability, the MTT (methylthiazoletetrazolium) test was performed as described in [32]. Absorbance was measured at 550 nm using a microplate spectrophotometer. The mean of the absolute absorbance values of the treated samples was divided by the absolute absorbance of the control samples and expressed as the % of cell viability. Caspase 3 activity was analysed using the Caspase-3 Fluorometric Assay Kit (Perbio Science, Belgium) according to the manufacturer's instructions. Cells ($2 \times 10^5$) were lysed in a buffer containing 10 mM Tris (pH 7.5), 130 mM NaCl, 1% Triton X-100 (v/v), 10 mM NaPi, 10 mM NaPPi; then 50 μg of protein was analysed. Analysis of apoptosis by propidium iodide incorporation was performed using permeabilised cells by flow cytometric analysis. Cells ($2 \times 10^4$) were harvested 24 h after the addition of doxorubicin to the cultures, washed in phosphate-buffered solution (PBS) and resuspended in 500 μl of a solution containing 0.1% sodium citrate (w/v), 0.1% Triton X-100 (v/v) and 50 μg/ml propidium iodide (Sigma Chemical Co., Gallarate, Italy). Following incubation at 4 °C for 30 min in the dark, cell nuclei were analysed using a Becton Dickinson FACScan flow cytometer. Cellular debris was excluded from the analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale. The percentage of the elements in the hypodiploid region was calculated.

### 2.3. IKK immunoprecipitation and kinase assay

Melanoma cells ($1 \times 10^6$), preincubated or not with rapamycin (100 ng/ml), were cultured in the absence or presence of doxorubicin (10 μM). After a 5-h incubation, cells were harvested, washed and lysed in a buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1 mM Na3V04, 20 mM β-glycerophosphate, 1 mM NaF and a complete protease inhibitor mixture (Roche). After a 30-min incubation on ice, the lysates were cleared by centrifugation for 15 min at 4 °C at 14 000 rotations per minute (rpm). Endogenous IKK was immunoprecipitated using an anti-IKK antibody H470 (Santa Cruz Biotechnology) plus protein A-

agarose and the kinase activity was assayed using glutathione-*S*-transferase (GST)–IκB (Santa Cruz Biotechnology) as a substrate, as described in [33]. Briefly, 500 μg of immunoprecipitate was incubated with kinase buffer (20mM HEPES, pH 7.4, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 1 mM Na3V04) in the presence of 0.1 mM adenosine triphosphate (ATP), [γ-$^{32}$P]ATP and GST–IκB. After a 10-min incubation at 37 °C, the reactions were terminated by boiling in sodium dodecylsulphate (SDS) sample buffer, and the products were resolved by 10% SDS–polyacrylamide (w/v) gel electrophoresis. Phosphorylated proteins were detected by autoradiography.

### 2.4. Cell lysates and Western blotting analysis

For detection of IkBα, cytosolic extracts were obtained from $1 \times 10^6$ cells resuspended in 100 μl of lysis buffer (10 mM HEPES, pH 7.9, 1 mM ethylene diamine tetra acetic acid (EDTA), 60 mM KCl, 1 mM DTT, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 50 μg/ml antipain, 40 μg/ml bestatin, 20 μg/ml chymostatin, 0.2% (v/v) Nonidet P-40) for 15 min in ice. For detection of Bcl-2, c-IAP1 and FKBP51, whole cell lysates were prepared by homogenisation in modified RIPA buffer (150 mM sodium chloride, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100 (v/v), 1% sodium deoxycholic acid (w/v), 0.1% SDS (w/v), 5 μg/ml of aprotinin and 5 μg/ml of leupeptin). Cell debris was removed by centrifugation. Protein concentrations were determined using the Bio-Rad protein assay. The cell lysate was boiled for 5 min in 1× SDS sample buffer (50 mM Tris–HCl pH 6.8, 12.5% glycerol, 1% SDS (w/v), 0.01% bromophenol blue (w/v)) containing 5% β-mercaptoethanol, run on 10% SDS (w/v) polyacrylamide gel, transferred onto a membrane filter (Cellulosenitrate, Schleider and Schuell) and finally incubated with the primary antibody. Anti-human-antibodies against IκB α (a rabbit polyclonal antibody from Santa Cruz Biotechnology), Bcl-2 (a mouse monoclonal antibody from Santa Cruz Biotechnology), c-IAP1 (a mouse monoclonal antibody from BD Pharmingen), FKBP51 (a rabbit polyclonal antibody from Abcam Limited, UK) were used at a dilution of 1:500. After a second incubation with peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) or anti-mouse IgG (Santa Cruz Biotechnology), the blots were developed using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

### 2.5. Nuclear extracts, electrophoretic mobility shift assay (EMSA), and oligonucleotides

Cell nuclear extracts were prepared from $1 \times 10^6$ cells by homogenisation of the cell pellet in two volumes of 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 10% glycerol (v/v). Nuclei were centrifuged at 1000*g* for 5 min, washed and resuspended in two volumes of the above specified solution. KCl (3M) was added until the concentration reached 0.39 M KCl. Nuclei were extracted at 4 °C for 1 h and centrifuged at 10 000*g* for 30 min. The supernatants were clarified by centrifugation and stored at −80 °C. Protein concentrations were determined using the Bradford method. The NF-κB consensus 5′-CAACGG-CAGGGGAATCTCCCTCTCCTT-3′ oligonucleotide [34] was end-labelled with [γ-$^{32}$P] adenosine triphosphate (ATP) (Amersham) using a polynucleotide kinase (Roche). End-labelled DNA fragments were incubated at room temperature for 20 min with 5 μg of nuclear protein, in the presence of 1 μg poly(dI–dC), in 20 μl of a buffer consisting of 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 5% glycerol (v/v). In supershifting experiments, rabbit antibodies against the C-terminal peptide (529–551) of human p65 or against the N-terminal peptide (1–21) of p105/p50 (kindly provided by Dr. Warner C. Greene, Gladstone Institute of Virology and Immunology, San Francisco, CA) were added to the incubation mixture. Protein–DNA complexes were separated from the free probe on a 6% polyacrylamide (w/v) gel run in 0.25× Tris borate buffer at 200 mV for 3 h at room temperature. The gels were dried and exposed to X-ray film (Kodak AR).

### 2.6. Transfection of siRNA

Twenty-four hours before transfection with short-interfering RNA (siRNA) oligonucleotide corresponding to the target sequence 5′-ACCUAAUGCUGAGCU-DAU-3′ of the sense-strand of human FKBP51 (Dharmacon Research Inc.) or a scrambled duplex as a control (Dharmacon Research Inc.), cells were seeded into six-well plates in medium without antibiotics at a concentration of $2.5 \times 10^5$/ml, to obtain 30–50% confluence at the time of transfection. The siRNA or the scrambled oligo were transfected at a final concentration of 50 nM using Oligofectamine (Invitrogen) according to the manufacturer's recommendations. After 3 days, the cells were processed for Western blotting analysis.

### 2.7. Statistical analysis

Statistical analysis of the results was performed by the Student's *t* test.

## 3. Results

### 3.1. Rapamycin enhanced doxorubicin-induced apoptosis in human melanoma cells

Melanoma tumours are poorly responsive to the cytotoxic effects of doxorubicin [30]. We found that

rapamycin greatly enhanced the amount of cell death induced by the anthracycline in melanoma cells. Analysis of cell viability by the MTT assay showed that 84.4 ± 6.1% of doxorubicin-treated cells were still alive after 16 h of incubation. As shown in Fig. 1, Panel (a), rapamycin alone did not substantially affect melanoma cell survival, while in the presence of doxorubicin, the macrolide agent reduced cell viability to 56.3 ± 0.1%. Analysis of caspase 3 activity confirmed that rapamycin alone did not activate the apoptotic process in melanoma cells. Indeed, the levels of active caspase 3 in cells cultured in RPMI with or without rapamycin were comparable and <6 U/ml. Fig. 1, Panel (b) shows the kinetics of caspase 3 activation in cells cultured with doxorubicin, with or without rapamycin. We found that the anthracycline drug alone produced an eightfold increase in the basal caspase 3 activity, while the addition of rapamycin produced a further 25% increase after a 8-h incubation. Analysis of apoptosis by propidium iodide incorporation and flow cytometry, revealed that doxorubicin induced 34.3% ± 8.2 apoptosis after 24 h in the absence of rapamycin and 55.9% ± 9.3 when the macro-

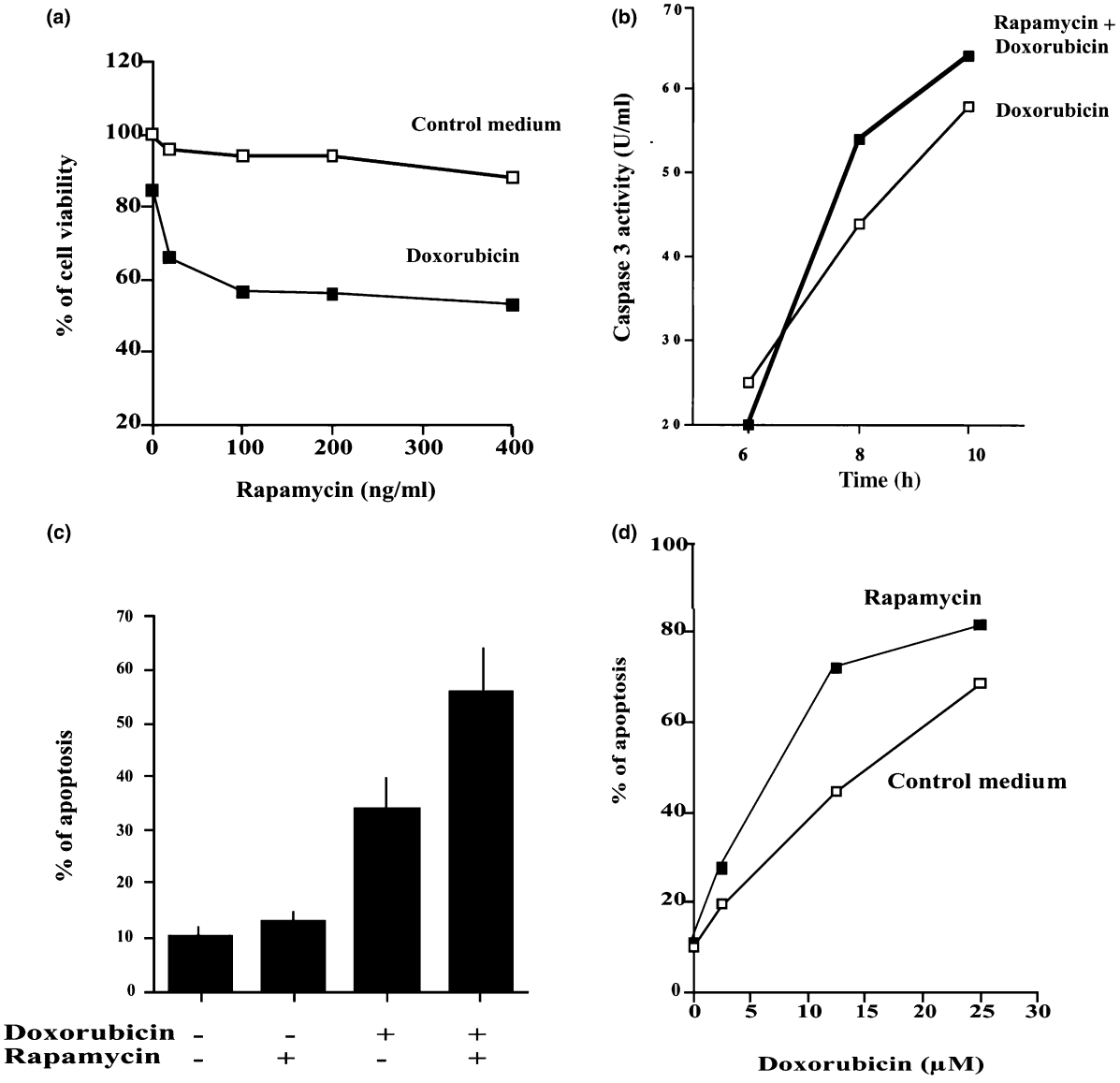


Fig. 1. Rapamycin enhances doxorubicin-induced apoptosis in human melanoma cells. (Panel (a)) Cells, preincubated or not with rapamycin at the indicated doses, were cultured in the absence or the presence of 10 μM doxorubicin. After a 16-h incubation, cell viability was analysed by the methylthiazoletetrazolium (MTT) assay. (Panel (b)) Cells, preincubated or not with rapamycin (100 ng/ml), were cultured with 10 μM doxorubicin. After 6, 8 and 10 h, cells were harvested and the lysates obtained were analysed using a fluorometric assay to detect caspase 3 activation. (Panel (c)) Cells, preincubated or not with rapamycin (100 ng/ml), were cultured in the absence or the presence of 10 μM doxorubicin. After a 24-h incubation, cells were harvested and an analysis of the amount of apoptosis was performed by propidium iodide incorporation and flow cytometry. (Panel (d)) Cells, preincubated or not with rapamycin (100 ng/ml), were cultured in the absence or the presence of doxorubicin at the indicated doses. After 24 h of incubation, cells were harvested and an analysis of apoptosis was performed by propidium iodide incorporation and flow cytometry.

lide was added to the cultures ($P = 0.042$) (Fig. 1, panel (c)). A dose–response curve of doxorubicin-induced apoptosis and its modulation by rapamycin is shown in Fig. 1, panel (d). Results represented in Fig. 1, were obtained in four different experiments, each performed in triplicate.

### 3.2. Doxorubicin induced NF-κB/Rel nuclear activation in human melanoma cells

The response of the cells to anthracyclines is modulated by activation of NF-κB/Rel transcription factors [31]. Western blots and EMSA verified that doxorubicin induced, IκB α degradation and NF-κB/Rel nuclear translocation, in the melanoma cells (Fig. 2, panels (a) and (b)). IκBα degradation was detected after a 3-h incubation and was complete after 5 h. Analysis of the NF-κB/Rel complexes by supershifting showed mainly p50/p65 heterodimers (Fig. 2, panel (b)).

### 3.3. Rapamycin inhibited doxorubicin-induced NF-κB/Rel activation in human melanoma cells

To investigate if the pro-apoptotic effect of rapamycin on doxorubicin-induced apoptosis could be related to the inhibition of cell survival pathways governed by

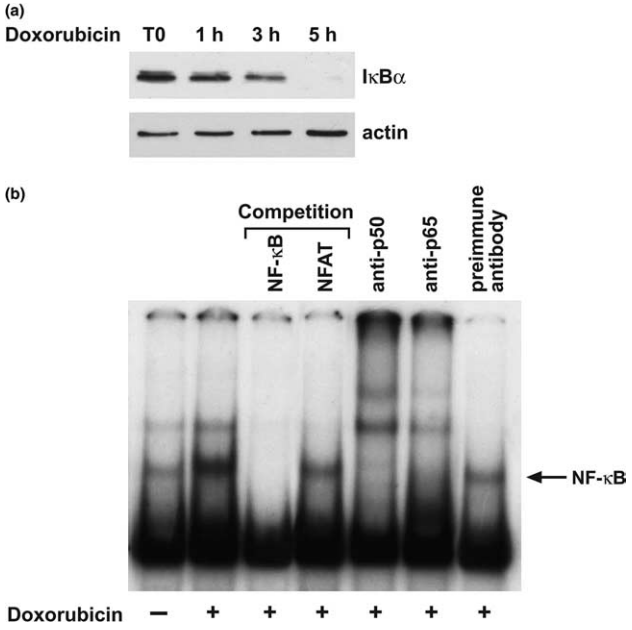


Fig. 2. Doxorubicin induces NF-κB/Rel activation in human melanoma cells. (Panel (a)) Western blotting analysis of IκB α protein. Cells were cultured in the presence of 10 μM doxorubicin and after 1-, 3-, 5-h of incubation were harvested and cytosolic extracts were obtained. (Panel (b)) electrophoretic mobility shift assay (EMSA) analysis of nuclear extracts obtained from human melanoma cells cultured in the absence or the presence of 10 μM doxorubicin for 5 h. A competition assay with the indicated cold oligos demonstrated the specificity of NF-κB band. Supershifting analysis showed the presence of p50/p65 heterodimers.

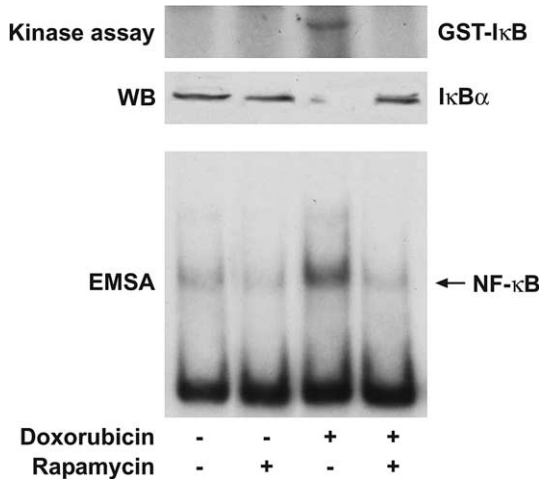


Fig. 3. Rapamycin inhibits doxorubicin-induced IκBα phosphorylation and degradation and NF-κB/Rel nuclear translocation. Melanoma cells, preincubated or not with rapamycin (100 ng/ml), were cultured in the absence or presence of 10 μM doxorubicin. After a 5-h incubation, cells were harvested and lysates were analysed using a IKK kinase assay to detect phosphorylated-IκBα, or by Western blotting to verify IkBα degradation. Nuclear extracts were also analysed by EMSA to detect NF-κB/Rel nuclear translocation.

NF-κB/Rel transcription factors, we first analysed the effect of rapamycin on the NF-κB/Rel nuclear activity induced by doxorubicin. We incubated cells, pretreated or not with rapamycin, in the absence or the presence of doxorubicin and after 5 h obtained nuclear- and cytosolic-extracts. Then, we analysed the catalytic activity of the IKK kinase complex using a kinase assay and, at the same time, investigated IκBα degradation and NF-κB/Rel nuclear translocation by Western blotting analysis and EMSA, respectively. Fig. 3 shows that rapamycin inhibited the phosphorylation of the IκB substrate, the degradation of IkB α and the nuclear translocation of NF-κB/Rel complexes induced by doxorubicin.

### 3.4. Rapamycin downmodulated the levels of Bcl-2 and c-IAP1

Among the NF-κB/Rel-regulated genes, Bcl-2 [24] and c-IAP1 [25] have been implicated in the resistance of tumour cells to the cytotoxic effects of doxorubicin [35,36]. For this reason, we investigated if rapamycin could sensitise melanoma cells to anthracycline-cytotoxicity through a decrease in the levels of these anti-apoptotic proteins. Western blotting analysis of cell lysates obtained after 6 h of culturing showed that doxorubicin upregulated Bcl-2 and c-IAP1 expression levels in melanoma cells, but to a lesser extent in the presence of rapamycin (Fig. 4). Although several reports suggest that anti-apoptotic members of Bcl-2-family could be stimulated through PI3k/Akt activation [37], analysis of phosphorylated-Akt at Thr308 or Ser473 did not reveal an increase of Akt activation following doxorubicin

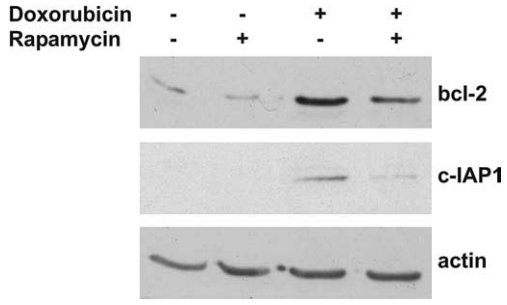


Fig. 4. Rapamycin counteracts the doxorubicin-induced increase in Bcl-2 and c-IAP1 expression levels Melanoma cells, preincubated or not with rapamycin (100 ng/ml), were cultured in the absence or presence of 10 μM doxorubicin. After a 6-h incubation, whole cell lysates were obtained and subjected to Western blotting for the detection of Bcl-2 and c-IAP1.

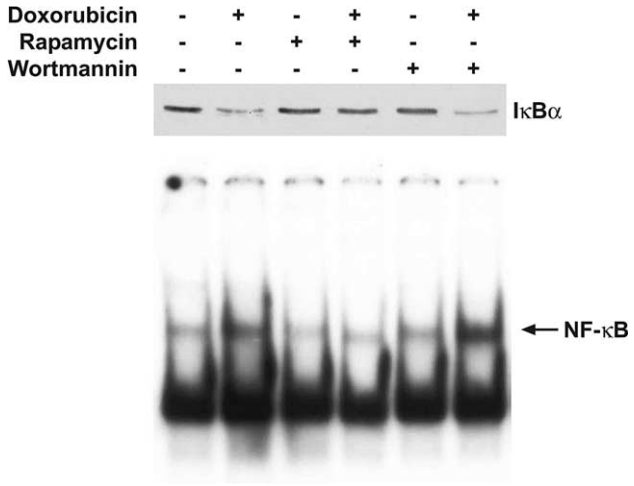


Fig. 5. Wortmannin does not inhibit doxorubicin-induced NF-κB/Rel activation. Melanoma cells, preincubated or not with rapamycin (100 ng/ml) or wortmannin (10 μM), were cultured in the absence or the presence of 10 μM doxorubicin. After a 5-h incubation, cells were harvested and cytosolic or nuclear extracts for Western blotting and EMSA analysis respectively, were obtained.

treatment (data not shown). Thus, their upregulation was apparently related to NF-κB/Rel activation. These results suggest that the doxorubicin-induced NF-κB/Rel activation in melanoma cells resulted in an increase in the expression of anti-apoptotic genes, namely Bcl-2 and c-IAP1, and this was responsible for the poor cytotoxic effects of the drug. By contrast, rapamycin, by counteracting the NF-κB/Rel nuclear translocation, decreased the levels of these proteins, thereby enhancing apoptosis, in these cells.

### *3.5. PI3K/Akt pathway inhibition was not involved in the NF-κB/Rel downmodulation produced by rapamycin*

To verify if the inhibition of NF-κB/Rel activation was due to inhibition of the PI3K/Akt/mTOR pathway, we investigated if the PI3K inhibitor wortmannin was able to antagonise IκB α degradation and NF-κB/Rel nuclear translocation in response to doxorubicin. Fig. 5 shows that wortmannin could not inhibit doxorubicin-induced NF-κB/Rel nuclear translocation and IkB α degradation. Thus, we conclude that the downmodulation of NF-κB/Rel activity is independent of the inhibition of the phosphatidylinositol triphosphate kinase pathway. These findings suggest that rapamycin could synergise with anti-cancer drugs that activate NF-κB/Rel transcription factors in PTEN-positive tumours.

### *3.6. The rapamycin-binding protein FKBP51, is expressed at high levels in melanoma cells and is involved in the NF-κB signalling pathway*

FKBP51 has been cloned in T lymphocytes, where it appears to be particularly abundant [39]. This immunophilin displays a peptidyl-prolyl-isomerase activity that has been shown to be essential for the function of IKK-α [21]. Rapamycin binds very specifically to this protein and inhibits the isomerase activity [39]. Fig. 6,

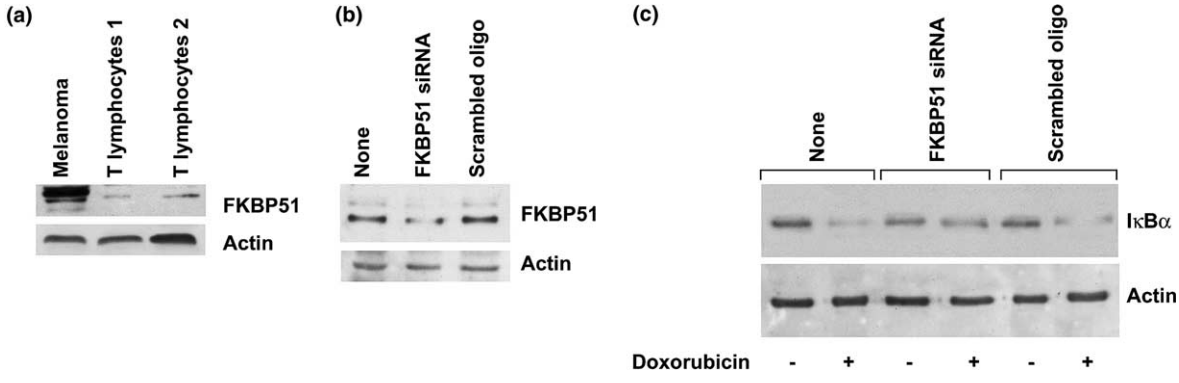


Fig. 6. FKBP51 is expressed at high levels in melanoma cells and is involved in the NF-κB signalling pathway (Panel (a)) Western blotting analysis of FKBP51 expression levels in cell lysates (20 μg) obtained from the melanoma cell line and two different preparations of purified primary T lymphocytes. (Panel (b)) Western blotting analysis of FKBP51 expression levels of cell lysates (2.5 μg) obtained from the melanoma cell line transfected or not with FKBP51 short interfering RNA (siRNA) or the scrambled oligo as a control. (Panel (c)) Western blotting analysis of IκB α expression levels of cells transfected with FKBP51 siRNA and cultured with or without doxorubicin (10 μM).

panel (a) shows that FKBP51 is expressed at high levels in melanoma cells in comparison to two different preparations of purified primary T lymphocytes. To investigate the role of FKBP51 in the NF-κB activation pathway, we downmodulated the immunophilin using a siRNA approach. As is shown in Fig. 6, panel (b), cells transfected with FKBP51 siRNA displayed a striking decrease in their protein expression levels, compared with cells incubated with control medium or transfected with a scrambled oligo. We then verified if doxorubicin was still able to induce IκBα degradation in the presence of low FKBP51 levels. Fig. 6, panel (c) shows that IκBα disappeared in control- or scrambled-oligo-transfected cells cultured with doxorubicin, according to its degradation, while it did not in the FKBP51 siRNA-transfected cells. These experiments suggest that FKBP51 is synthesised in melanoma cells and controls IKK activity in this tumour.

## 4. Discussion

Both the PI3k/Akt- and NF-κB/Rel pathways are involved in oncogenic processes and promote cell survival following stimuli which lead to cell death [12–14,25–29]. The inhibitory effect of rapamycin on the downstream effectors of the phosphatidylinositol triphosphate pathway is well known [1–13]. Herein, we demonstrated that rapamycin downregulated the NF-κB/Rel activation induced by doxorubicin, independently of PI3k/Akt inhibition. Furthermore, we showed that the macrolide agent was able to sensitise poorly responsive human melanoma cells [30] to anthracycline-triggered apoptosis, apparently by antagonising the induction of the anti-apoptotic genes, Bcl-2 and c-IAP1, that are under NF-κB control. Several reports suggest that anti-apoptotic members of the Bcl-2-family can be upregulated by PI3k/Akt [37]. However, analysis of phosphorylated-Akt at Thr308 or Ser473 did not reveal an increase of phospho-Akt following doxorubicin stimuli (data not shown), thus the increased Bcl-2 and c-IAP1 protein expression levels appeared to be related to NF-κB/Rel activation. A critical step in the activation of NF-κB is the phosphorylation of IκB proteins by the IKK complex targeting them for degradation by the proteasome [38]. We analysed the pathway of doxorubicin-induced NF-κB activation, from I κBα phosphorylation to nuclear translocation of the transcription factors and found that rapamycin inhibited IKK kinase activity. Recently, an integrated approach of proteomic NF-κB pathway mapping detected new components involved in this signal transduction pathway [21]. Among them, FKBP51, with peptidyl-prolyl-isomerase activity, was identified as an important IKK-α cofactor. Furthermore, functional analysis with RNA interference revealed that the immunophilin was essential for the overall signalling process leading to NF-κB nuclear translocation [21]. We found high levels of expression of FKBP51 in melanoma cells. In addition, we confirmed the involvement of this immunophilin in the control of IKK activity. Since rapamycin binds with high affinity to FKBP51 [39], and specifically inhibits its isomerase activity [39], it is reasonable to assume that it can affect IKK catalytic function through this mechanism. The IKK complex is an important target for therapeutic intervention, since it represents the converging point for the activation of NF-κB by a broad spectrum of stimuli that sustain cell survival and tumour progression. Indeed, a new class of drugs, based on molecules that inhibit the NF-κB signalling pathway have now been developed for the treatment of diseases which result from abnormal cell proliferation and cell death, including cancer [40]. Our findings suggest that rapamycin, when associated with drugs that induce NF-κB activation, could improve the effectiveness of treatments, even for aggressive tumours, such as melanoma, that are often resistant to standard anti-cancer therapies. Our observation that rapamycin acts through IKK is a novel mechanism that should increase interest in this molecule as an anti-cancer agent. Furthermore, our findings that the effect on NF-κB was independent of PI3k/Akt inhibition suggests that the drug could synergise with chemotherapeutic drugs in PTEN-positive tumours.

## Conflict of Interest Statement

None declared.

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