Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54(nrb), an event linked to G2/M arrest

Journal Item

JOURNAL OF PROTEOMICS XX (2008) XXX–XXX

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54^{nrb}, an event linked to G2/M arrest☆

Pedro Casado[a], *Miguel A. Prado*[a], *Pedro Zuazua-Villar*[a], *Eva del Valle*[a], *Noelia Artime*[a], *Lucía Cabal-Hierro*[a], *Patricia Rupérez*[b], *Alma L. Burlingame*[b], *Pedro S. Lazo*[a], *Sofía Ramos*[a,*]

[a]*Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo, 33071, Oviedo, Spain*

[b]*Mass Spectrometry Facility, Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0446, USA*

ARTICLE DATA

Article history:
Received 7 June 2008
Accepted 8 September 2008

Keywords:
2D–PAGE
p54^{nrb}
Nano-LC-ESI-Q-ToF
Vincristine
Paclitaxel
Phosphorylation

ABSTRACT

Microtubule interfering agents (MIAs) are anti-tumor drugs that inhibit microtubule dynamics, while kinesin spindle protein (KSP) inhibitors are substances that block the formation of the bipolar spindle during mitosis. All these compounds cause G2/M arrest and cell death. Using 2D–PAGE followed by Nano-LC-ESI-Q-ToF analysis, we found that MIAs such as vincristine (Oncovin) or paclitaxel (Taxol) and KSP inhibitors such as S-tritil-L-cysteine induce the phosphorylation of the nuclear protein p54^{nrb} in HeLa cells. Furthermore, we demonstrate that cisplatin (Platinol), an anti-tumor drug that does not cause M arrest, does not induce this modification. We show that the G2/M arrest induced by the MIAs is required for p54^{nrb} phosphorylation. Finally, we demonstrate that CDK activity is required for MIA-induced phosphorylation of p54^{nrb}.

1. Introduction

Microtubule dynamics is an important process for many cellular events, especially for cell division where the microtubule architecture suffers intense modifications. This implication in cell division makes microtubules a relevant target for anti-cancer drugs [1].

Microtubule interfering agents (MIAs) are compounds that bind to tubulin and block microtubule dynamics [2]. This causes JNK activation [3], Bcl-2 phosphorylation [4], G2/M arrest and cell death [3,5]. Nowadays, the most used MIAs for cancer treatment are vinca alkaloids (VAs) and taxanes [6].

VAs are drugs derived from the periwinkle *Catharanthus roseus*. This group comprises natural molecules such as

☆ This work was supported by grants from the Fondo de Investigación de la Seguridad Social (FISS-05-PI-042664) and the Ministerio de Educación y Ciencia (SAF2006-09686). P.C., M.A.P., E.d.V, L.C.H. and N.A. are recipients of fellowships from FICYT, and P.Z.V. is recipient of a fellowship from the IUOPA. The Instituto Universitario de Oncología del Principado de Asturias (IUOPA) is supported by the Obra Social Cajastur-Asturias.

Abbreviations: MIAs, Microtubule Interfering Agents; JNK, c-Jun-NH2-Terminal Kinase; λ-PPase, λ-Phosphatase; VAs, Vinca Alkaloids; PSF, PBT-associated splicing factor; NonO, Non-POU domain-containing octamer-binding protein; KSP, Kinesin spindle protein.

* *Corresponding author*. Tel.: +34 985102764; fax: +34 985104213.
E-mail address: sramos@uniovi.es (S. Ramos).

1874-3919/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jprot.2008.09.001

vincristine and vinblastine and semisynthetic molecules such as vindesine and vinorelbine [7]. These agents inhibit microtubule dynamics by binding to the interface of two tubulin heterodimers. This interaction forms a wedge that blocks the polymerization of microtubules [8].

Taxanes are drugs derived from the trees *Taxus baccata* and *Taxus brevifolia*. This group comprises the natural molecule paclitaxel and the semisynthetic molecule docetaxel. These agents block microtubule dynamics by binding to the taxane binding domain of β-tubulin. This event stabilises the microtubule network and inhibits its depolymerization [2].

In the last few years, there has been an intense search of new targets for cancer treatment. One of these novel targets is the mitotic specific kinesin (KSP) which is motor protein that is required for the formation of the bipolar spindle during mitosis [9]. Specific KSP inhibitors have been developed. These compounds bind to an allosteric site adjacent to loop 5 that is not present in other related kinesins. These drugs also induce G2/M arrest and cell death and some of them, such as ispinesib, are in phase II of clinical trials [10].

The protein p54^{nrb}, also known as NonO, is an abundant nuclear component that binds DNA and RNA. This conserved factor is associated with the highly similar protein PSF in several macromolecular complexes that are implicated in many nuclear processes [11]. Thus, these proteins regulate transcription [11] and are related with the coupling of transcription and splicing [12]. They also cause the nuclear retention of defective mRNAs [13], increase the DNA topoisomerase I activity [14] and facilitate the formation of the preligation complex during non homologous end joining repair [15]. Furthermore, this protein is implicated in cell differentiation [16,17] and its silencing in breast cancer is associated with loss of estrogen receptor alpha expression and increase of tumor-size [18].

By using 2D–PAGE and Nano-LC-ESI-Q-ToF analysis we have determined that MIAs and the KSP inhibitor S-tritil-L-cysteine (STLC) induce the phosphorylation of the nuclear protein p54^{nrb} while cisplatin (another anti-tumor drug that does not induce G2/M arrest) does not induce this modification. We demonstrate that the G2/M arrest caused by MIA is required for p54^{nrb} phosphorylation and CDK activity is required for this modification to take place.

2. Materials and methods

2.1. *Cell culture and treatments*

HeLa and HEK 293 cells were propagated in phenol-red DMEM (Cambrex) containing 100 μg/mL gentamicin and 10% of heat inactivated fetal bovine serum (FBS) (Cambrex). For experiments, cells were transferred to phenol-red free DMEM containing 0.5% of charcoal/dextran-treated FBS, 100 μg/mL gentamicin and 4 mM L-glutamine. HeLa cells were kept in this medium for three days and treated while HEK 293 cells were transferred to this medium and treated at the same time. Cells were incubated with vincristine (Sigma), vinblastine (Sigma), paclitaxel (Sigma), docetaxel (Fluka) and aphidicolin (Sigma) dissolved in ethanol and with cisplatin (Sigma), S-tritil-L-cysteine (Calbiochem), and roscovitine (Calbiochem) were dissolved in DMSO. The final concentrations of ethanol and DMSO were 0.1%.

2.2. *Flow cytometry*

Cells were collected by trypsinization and incubated sequentially, according to Vindelov's technique in 300 μL of buffer A (0.5 mM Tris–HCl pH 7.6, 0.1% Nonidet P-40 v/v, 3.4 mM trisodium citrate, 1.5 mM spermine, 30 μg/mL trypsin from Sigma) for 10 min, in 250 μL of buffer B (0.5 mM Tris–HCl pH 7.6, 0.1% Nonidet P-40 v/v, 3.4 mM trisodium citrate, 1.5 mM spermine, 500 μg/mL trypsin inhibitor from Sigma, 100 μg/mL RNase A from Sigma) for 10 min and in 250 μL of buffer C (0.5 mM Tris–HCl pH 7.6, 0.1% Nonidet P-40 v/v, 3.4 mM trisodium citrate, 4.83 mM spermine, 416 μg/mL propidium iodide) for 10 min. Cell cycle was analyzed in a FACscan flow cytometer (Becton Dickinson) using ModFYT software.

2.3. *Western blot analysis*

Cell extracts were obtained in Laemmli buffer, heat denatured and 5 to 10 μg of protein were electrophoresed on a 15% SDS–PAGE. After electrophoresis, proteins were transferred to PVDF membranes (Millipore). Membranes were blocked with TBS/Tween-20 supplemented with 5% w/v non-fat milk for 1 h at room temperature, then incubated with primary antibody overnight at 4 °C, with secondary antibody for 1 h at room temperature, and developed with enhanced chemiluminescence reagents (GE-Healthcare). Anti-histone H3 phosphorylated at serine 10 (Santacruz, Cat. sc-8656), anti-β-actin (Sigma, Cat. A-5441), anti-rabbit peroxidase (Cell Signaling, Cat. 7074) and anti-mouse peroxidase (Sigma, Cat. A-9044) were used at 1:50,000, 1:20,000, 1:2000 and 1:10,000 dilution respectively.

2.4. *Two-Dimensional Polyacrylamide Gel Electrophoresis (2D–PAGE)*

2D–PAGE experiments were carried out as described previously [19]. Briefly, cells were solubilized in UTATH buffer [7 M urea, 2 M thiourea, 1% Amidosulfobetaine-14, 50 mM 2-Hydroxyethyl disulfide (HED), 0.5% IPG buffer pH 3–10 (Bio-Rad)], desalted with a desalting spin column (Pierce) and 60 to 100 μg of protein were loaded onto a strip holder. First dimension was run in 7 cm Immobiline™ DryStrips pH 3–11 (GE-Healthcare) for 12 h at 30 V, 250 Vh at 500 V, 500 Vh at 1000 V and 8000 Vh at 5000 V. For second dimension, strips were equilibrated in equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris pH 6.8, 2% SDS, 0.002% bromophenol blue w/v) and run in 10% polyacrylamide gels supplemented with 50 mM HED and 6 M urea. For Coomassie staining, gels were fixed with fixing-solution (20% methanol v/v, 10% acetic acid v/v) for 24 h, stained with Coomassie-solution (0.25% brilliant blue R250 w/v, 45% methanol v/v, 10% acetic acid v/v) for 2 h and distained with fixing-solution for 24 h. For Western analysis, proteins were transferred to PVDF membranes and processed as described previously. Anti-p54^{nrb} (BD Biosciences, Cat. 611278), and anti-mouse peroxidase (Sigma, Cat. A-9044) were used at 1:10,000 dilution.

2.5. *Trypsin digestion, mass spectrometry and bioinformatics analysis of data*

Gel spots were subjected to in-gel digestion (http://msfacility.ucsf.edu/ingel.html) with trypsin (porcine, side-chain

Please cite this article as: Casado P, et al, Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54^{nrb}, an event linked to G2/M arrest, J. Prot. (2008), doi:10.1016/j.jprot.2008.09.001

protected, Promega). Briefly, protein spots were washed twice with 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH4HCO3) and vacuum-dried. Then, gel pieces were rehydrated in 25 μl of digestion buffer (10 ng/μl trypsin in 25 mM NH4HCO3) for 10 min at 4 °C. The digestion was performed for 4 h at 37 °C. Tryptic peptides were extracted twice with 50% ACN and 5% formic acid. Extracted peptides were vacuum-dried and resuspended in 10 μl of 0.1% formic acid in water. The digests were separated by nanoflow liquid chromatography using a 100-μm×150-mm reverse-phase Ultra 120-μm C18Q column (Peeke Scientific, Redwood City, CA) at a flow rate of 350 nl/min in an Eksigent high performance liquid chromatography system equipped with a FAMOS autosampler (both Dionex-LC Packings, San Francisco, CA). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in ACN. Following equilibration of the column in 2% solvent B, approximately one-tenth of each digest (1 μl) was injected, and then the organic content of the mobile phase was increased linearly to 40% over 30 min and then to 50% in 3 min. The liquid chromatography elute was coupled to a QSTAR-ELITE tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, CA). In every cycle, a 0.5 s of MS acquisition was followed by a maximum of 1.5 s of collision-induced-dissociation (CID) acquisition for each of the 3 most intense multiply charged peaks that were not previously acquired. CID collision energy was automatically determined based upon peptide charge and mass to charge (m/z) ratio. Protein Prospector 4.25.4 software (UCSF/ San Francisco, CA) [20] was used to analyze the mass spectra. Initial peptide tolerances in MS and MS/MS modes were 200 ppm and 0.2 Da, respectively. The data were searched against Swiss Prot database from 2007.04.19. Trypsin was designated as protease and 1 missed cleavage was allowed. Oxidation of methionine, N-terminal acetylation, N-terminal pyroglutamate, and HED modified cysteine (+76 Da) were allowed as variable modifications.

2.6. *"In vitro" dephosphorylation assay*

Paclitaxel treated cells were lysed in UTATH. Once UTATH was removed using a Y-10 microcone (Millipore), proteins were

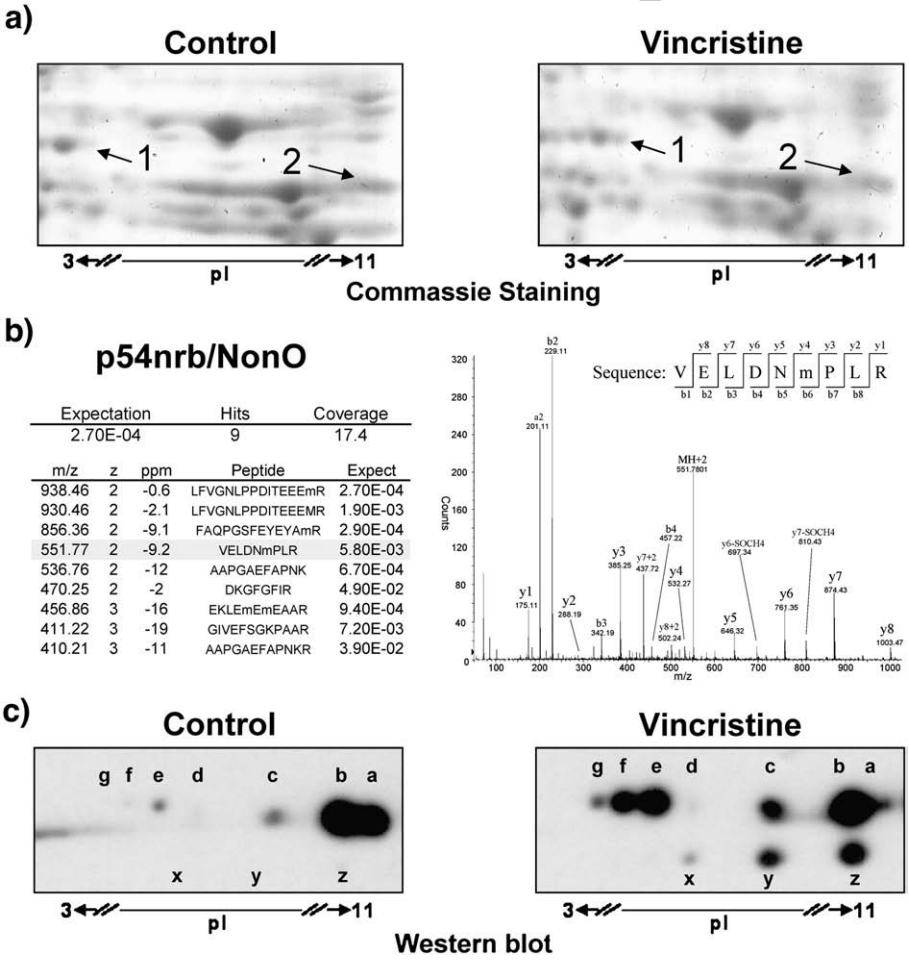

Fig. 1 – Vincristine effects over the nuclear factor p54^{nrb} in HeLa cells. Cells were treated with vehicle (control) or 1 μM vincristine for 24 h. a) Coomassie staining of a zone of a 2D–PAGE showing the spots of interest. b) Mass spectrometry analysis of spot 1. (m: oxidized methionine) c) Western blot analysis of a 2D–PAGE using specific antibodies against p54^{nrb}. As in a) only the region of the filter containing spots of interest is shown.

Please cite this article as: Casado P, et al, Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54^{nrb}, an event linked to G2/M arrest, J. Prot. (2008), doi:10.1016/j.jprot.2008.09.001

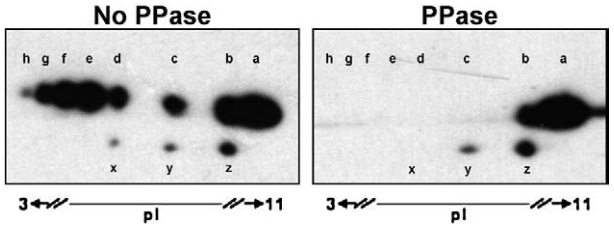

Fig. 2 – Study of p54^{nrb} phosphorylation state after treatment with vincristine. Cell extracts from vincristine-treated HeLa cells were incubated in the presence or the absence of λ PPase as indicated in Materials and methods. After incubation, cell extracts were analyzed by 2D–PAGE followed by Western blot using antibodies against p54^{nrb}.

recovered in water and quantified by Bradford assay. 560 μg of protein were dephosphorylated with λ-PPase (New England BioLabs) as described in the manufacturer protocol. After elimination of reaction buffer with a Y-10 microcone, proteins were recovered in UTATH and run in a 2D–PAGE as described above.

3. Results

3.1. *Identification of p54^{nrb} as a vincristine regulated protein*

To study the proteins altered upon vincristine treatment, HeLa cells were treated with either vehicle or 1 μM vincristine for 24 h. Protein expression after drug treatment was analyzed by 2D–PAGE. Observation of the electropherogram showed an up-regulated spot, named as 1, in vincristine-treated cells (Fig. 1a). For protein identification, spot 1 was excised from the gel and digested with trypsin. Then, the peptides obtained were analyzed by Nano-LC-ESI-Q-ToF. Finally, Protein Prospector analysis of mass spectrometry data determined that the protein in spot 1 corresponds to p54^{nrb} with an expectation value of 2.7×10^{-4} (Fig. 1b). Western blot analysis revealed 3 groups of p54^{nrb} forms. The most basic group comprises spots a and b; the most acidic group comprises spots d, e, f and g. Finally, the intermediate group comprises only spot c. Moreover, we observed that vincristine up-regulated the groups that comprise spots c to g. while had little effect over the basic group (Fig. 1c). Spot 1 in Coomassie staining corresponds to a form of p54^{nrb} included in the most acidic group (spots d to g). Spot 2 in Coomassie staining was also identified as p54^{nrb} by Ms/Ms and

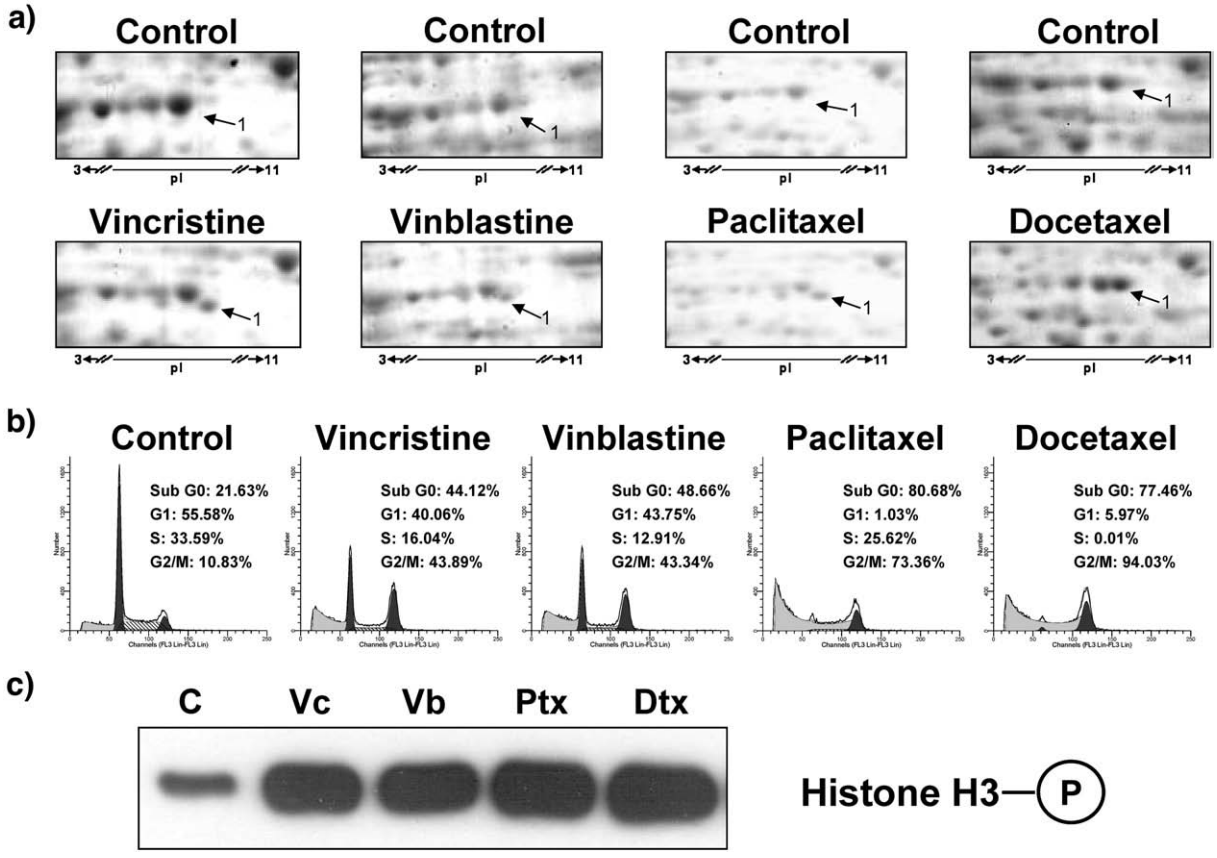

Fig. 3 – Effect of different MIAs on the phosphorylation of nuclear factor p54^{nrb}. HeLa cells were treated for 24 h with vehicle (control) or 1 μM vincristine (Vc), vinblastine (Vb), paclitaxel (Ptx) or docetaxel (Dtx) as indicated. a) Coomassie staining of 2D–PAGE. b) Flow cytometry analysis of treated cells. Percentage of cells at each cycle stage was calculated considering only alive cells c) Western blot analysis of treated cell extracts using specific antibodies against phosphorylated histone H3. For this analysis, β-actin levels were used as a loading control (data not shown).

Please cite this article as: Casado P, et al, Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54^{nrb}, an event linked to G2/M arrest, J. Prot. (2008), doi:10.1016/j.jprot.2008.09.001

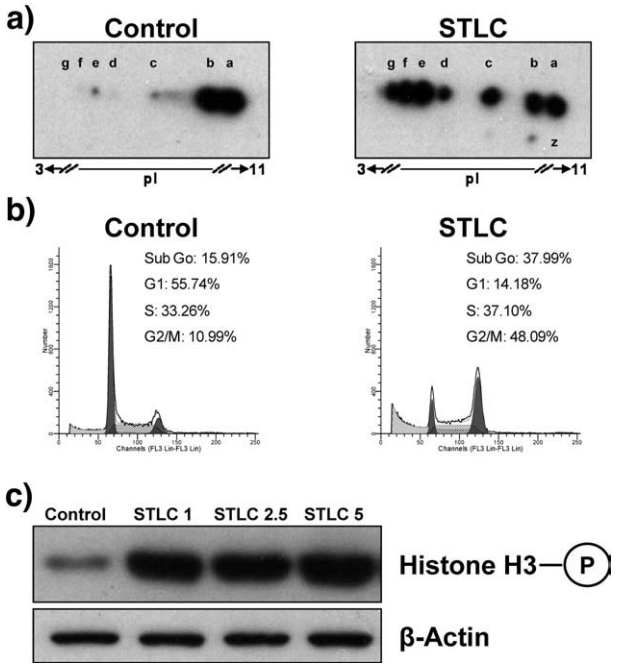

Fig. 4 – S-Tritil-L-cysteine effects on p54^{nrb} phosphorylation and cell cycle. HeLa cells were treated with vehicle (control) or 5 μM S-tritil-L-cystein (STLC) for 24 h. a) Western blot analysis of 2D–PAGE gels using antibodies against p54^{nrb}. b) Flow cytometry analysis of treated cells. c) Western blot analysis using antibodies against histone H3 phosphorylated. (STLC 1: 1 μM S-tritil-L-cysteine; STLC 2.5: 2.5 μM S-tritil-L-cysteine; STLC 5: 5 μM S-tritil-L-cysteine).

corresponds to a form included in the most basic group (spots a and b). Vincristine also induced 3 small forms of p54^{nrb} (Spots x, y and z). These spots probably correspond to caspase-processed forms of the protein, since they are not detected in the presence of caspase inhibitors (data not shown).

3.2. *Identification of some vincristine-induced spots of p54^{nrb} as phosphorylated forms*

To study if the vincristine-induced forms of p54^{nrb} are phosphorylated, an "in vitro" dephosphorylation assay was used. Thus, protein extracts from cells treated with 1 μM vincristine for 24 h were incubated in the presence or in the absence of λ-PPase as described in Materials and methods. Then, extracts were subjected to 2D–PAGE followed by Western blot analysis. Vincristine-induced forms of p54^{nrb} (c,d,e,f, g and h) were undetected after λ-PPase treatment, clearly indicating that these vincristine-induced forms are phosphorylated (Fig. 2). Spot x also disappeared after λ-PPase treatment and therefore, this form is also considered as phosphorylated (Fig. 2). The same extracts were run in a 2D–PAGE and gels were stained with Coomassie blue. As expected, spot 1 disappears after PPase treatment (data not shown).

3.3. *Effect of different microtubule interfering agents over the phosphorylation of p54^{nrb}*

Since vincristine is a microtubule interfering agent, we have analyzed whether other MIAs that induce G2/M arrest also induce the phosphorylation of this protein. Thus, HeLa cells were treated with vehicle or 1 μM of vincristine, vinblastine, paclitaxel, or docetaxel for 24 h. Then, cell extracts were subjected to 2D–PAGE and the gels were stained with Coomassie blue. The electropherograms showed that all these drugs induced the phosphorylation of p54^{nrb} (Fig. 3a). These phosphorylations were also confirmed by Western blot analysis (see Fig. 1 of supplementary material). As expected, the flow cytometry analysis of these cells showed that all the compounds used induced G2/M arrest and cell death (Fig. 3b). Furthermore, a Western blot analysis using antibodies against the molecular marker phospho-histone H3 indicated that the G2/M arrest induced by these agents is at M stage (Fig. 3c).

3.4. *Effect of the KSP inhibitor STLC over the phosphorylation of p54^{nrb}*

KSP inhibitors are anti-cancer drugs, still in clinical trials, that also induce G2/M arrest and cell death. Thus, we decided to analyse whether the KSP inhibitor S-tritil-L-cystein (STLC) is able to induce p54^{nrb} phosphorylation. HeLa cells were treated with vehicle (control) or 5 μM STLC for 24 h. Then, cells were analyzed by flow cytometry and cell extracts were subjected to Western blot analysis. As expected, STLC induced cell death (Fig. 4b) and M phase arrest (Fig. 4c). More interestingly,

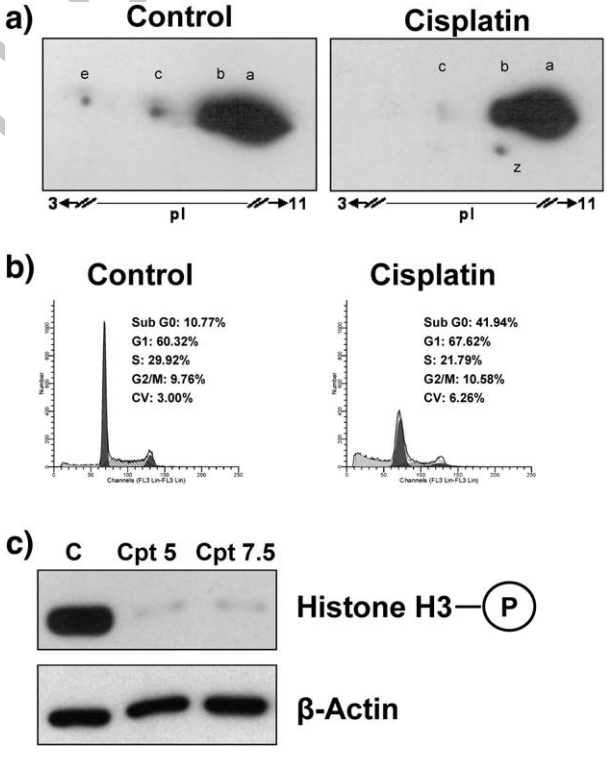

Fig. 5 – Cisplatin effects on p54^{nrb} phosphorylation and cell cycle. HeLa cells were treated with vehicle (control) or 7.5 μg/mL cisplatin for 24 h. a) Western blot analysis of 2D–PAGE gels using antibodies against p54^{nrb}. b) Flow cytometry analysis of treated cells. c) Western blot analysis using antibodies against histone H3 phosphorylated (C: control; Cp 5: 5 μg/mL cisplatin; Cp 7.5: 7.5 μg/mL cisplatin).

Please cite this article as: Casado P, et al, Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54^{nrb}, an event linked to G2/M arrest, J. Prot. (2008), doi:10.1016/j.jprot.2008.09.001

STLC induced the phosphorylation of p54^{nrb}. Furthermore, STLC also induced the smaller form z of the protein (Fig. 4a).

3.5. *Effect of cisplatin over p54^{nrb} phosphorylation*

Since all tested agents that induce p54^{nrb} phosphorylation also induce M arrest and cell death, we analyzed whether other anti-tumor drugs that induce cell death but not M arrest also trigger this modification. To study this premise, we have used cisplatin, a drug that induces DNA damage. Thus, we treated HeLa cells with vehicle or 7.5 μg/mL cisplatin for 24 h. Flow cytometry analysis confirmed that cisplatin causes cell death (Fig. 5b), while Western blot analysis using the specific marker histone H3 phosphorylated at serine 10, showed that this drug does not induce M arrest (Fig. 5c). Moreover, cisplatin caused an increase of the CV coefficient, indicating DNA damage [21] (Fig. 5b). 2D–PAGE analysis demonstrated that cisplatin does not trigger p54^{nrb} phosphorylation. Rather, it appears to downregulate the phosphorylated forms of this protein (Fig. 5a). This downregulation is coupled to a reduction in the number of M phase cells (Fig. 5c). Moreover, we observed that cisplatin treatment induced the smaller form z of p54^{nrb}. As mentioned above, this is probably a caspase-processed form. It has been recently demonstrated that during cisplatin induced cell death, p54^{nrb} is processed by caspases [22].

3.6. *p54^{nrb} phosphorylation induced by MIAs requires G2/M arrest*

Since only the drugs that produce G2/M arrest also induce p54^{nrb} phosphorylation, we analyzed if the G2/M arrest is necessary for the induction of phosphorylation. To check this option, we used arrested cells at the beginning of S phase, by using the DNA polymerase inhibitor aphidicolin. Thus, HeLa cells were treated with vehicle, 2 μg/mL aphidicolin, 1 μM vincristine, 1 μM paclitaxel or the combination of 2 μg/mL aphidicolin with 1 μM vincristine or 1 μM paclitaxel for 24 h.

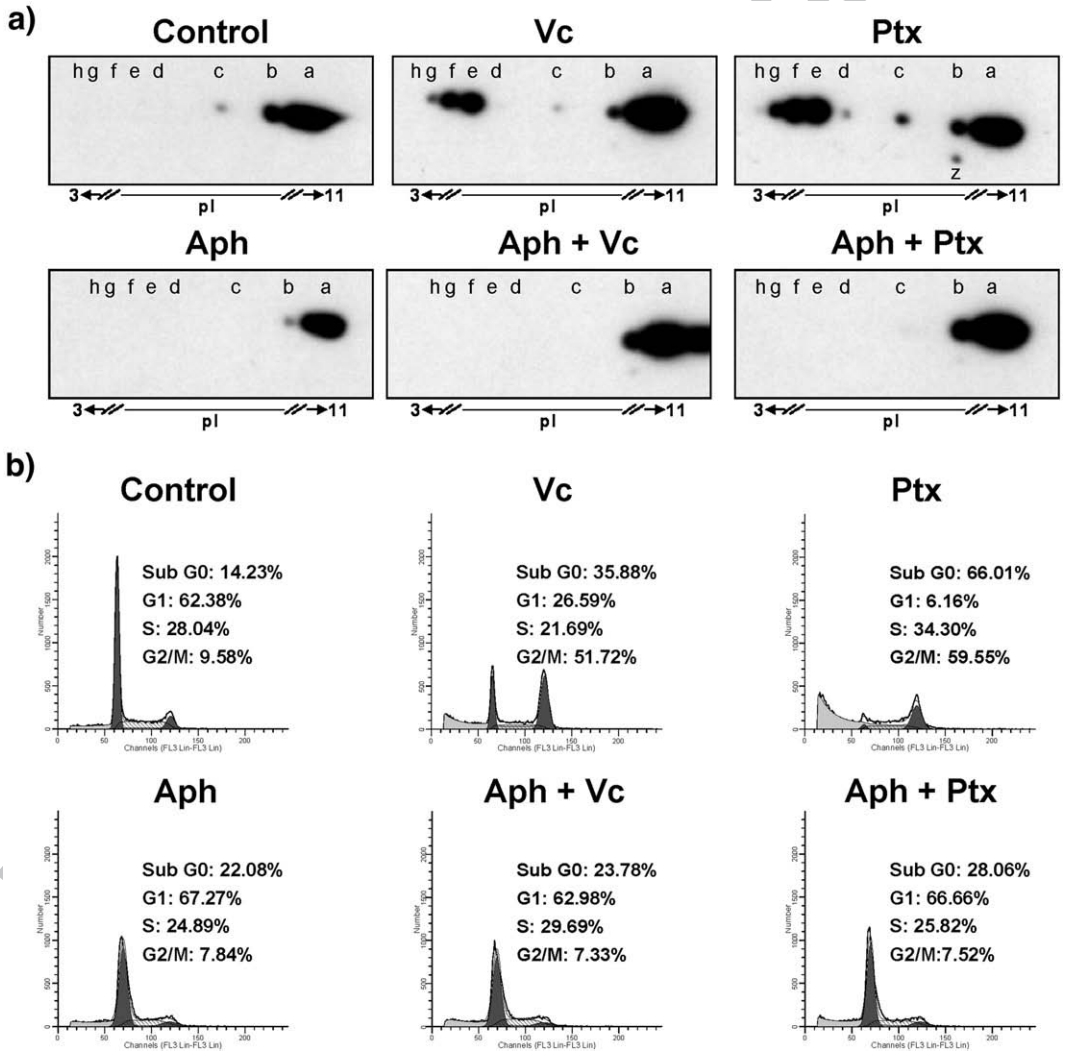

Fig. 6 – Effect of aphidicolin-induced cell cycle arrest over MIA-induced phosphorylation of p54^{nrb}. HeLa cells were treated with vehicle (control), 1 μM vincristine (Vc), 1 μM paclitaxel (Ptx), 2 μg/mL aphidicolin (Aph) or the combination of aphidicolin with 1 μM vincristine (Aph + Vc) or 1 μM paclitaxel for 24 h (Aph + Ptx). Aphidicolin was added 24 h before MIA treatment. a) Western blot analysis of 2D–PAGE gels using antibodies against p54^{nrb}. b) Flow cytometry analysis of treated cells.

Please cite this article as: Casado P, et al, Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54^{nrb}, an event linked to G2/M arrest, J. Prot. (2008), doi:10.1016/j.jprot.2008.09.001

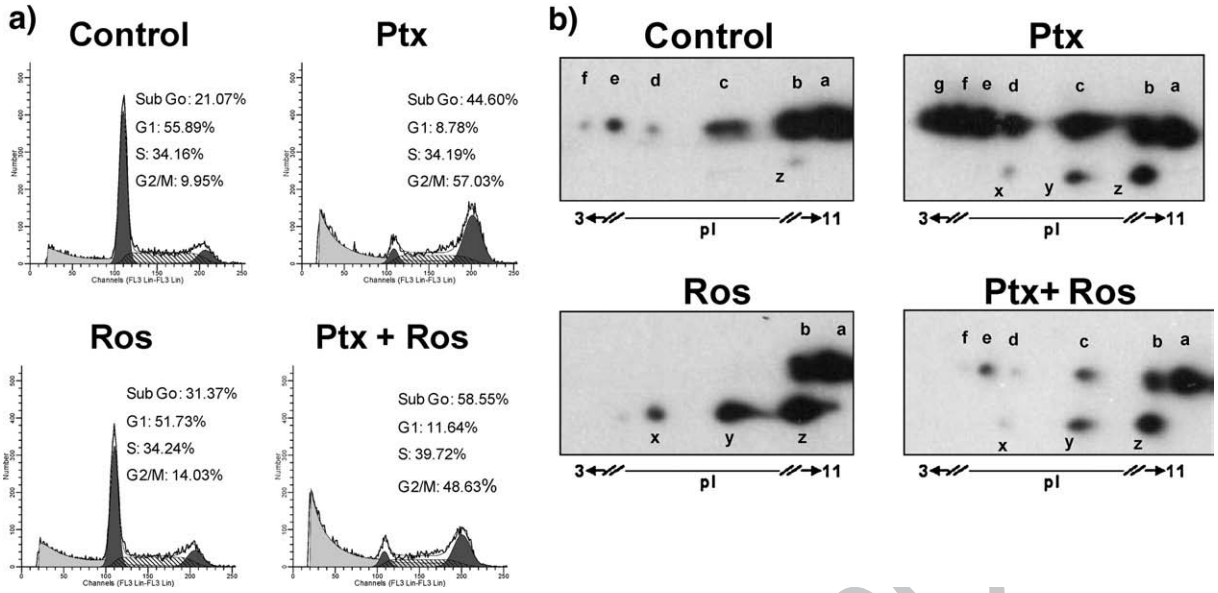

Fig. 7 – Effect of roscovitine-induced inhibition of CDK1 over MIA-induced phosphorylation of p54^{nrb}. HeLa cells were treated with vehicle (control), 1 μM paclitaxel (Ptx), 50 μM roscovitine (Ros) or the combination of 1 μM paclitaxel and 50 μM roscovitine (Ptx + Ros) for 6 h. Paclitaxel was added 24 h before roscovitine treatment. a) Flow cytometry analysis of treated cells. b) Western blot analysis of the treated extracts using antibodies against p54^{nrb}.

Aphidicolin was added 24 h prior to MIA treatment. Then, cell extracts were subjected to 2D–PAGE followed by Western blot. Both MIAs induced the phosphorylation p54^{nrb}. Furthermore, paclitaxel also induced the form z of the protein. More interestingly, aphidicolin precluded the phosphorylation of p54^{nrb} induced by vincristine or paclitaxel (Fig. 6a). The treated cells were also analyzed by flow cytometry. Vincristine and paclitaxel induced G2/M arrest, while in cells pretreated with aphidicolin this induction did not occur (Fig. 6b). These results clearly show an association between the induction of G2/M arrest and the p54^{nrb} phosphorylation.

3.7. *CDK activity is required for MIA-induced phosphorylation of p54^{nrb}*

It has been described that MIAs induce CDK activity and that one of these family of kinases, CDK1, phosphorylates p54^{nrb} during mitosis. Thus, we decided to study whether CDK activity is required for MIA-induced phosphorylation of p54^{nrb}, using the CDK inhibitor roscovitine. HeLa cells were treated with vehicle, 1 μM paclitaxel, 50 μM roscovitine or the combination of 1 μM paclitaxel and 50 μM roscovitine. Paclitaxel was added 24 h before roscovitine. Flow cytometry analysis revealed that paclitaxel induces G2/M arrest and cell death, while no relevant effect was detected after roscovitine treatment (Fig. 7a) The results demonstrate that paclitaxel induces the phosphorylated forms d to g (Fig. 7b). This drug also induces the spots x, y and z which correspond to processed forms of the protein (Fig. 7b). Roscovitine treatment induced p54^{nrb} processing but did not affect its phosphorylation (Fig. 7b). Interestingly, the addition of roscovitine reduced the amount of the phosphorylated forms of p54^{nrb} that are up-regulated by paclitaxel (forms d, e, f, g and x) (Fig. 7b). These data indicate that CDK activity is needed in the signalling pathway that triggers the MIA-induced phosphorylation of p54^{nrb}.

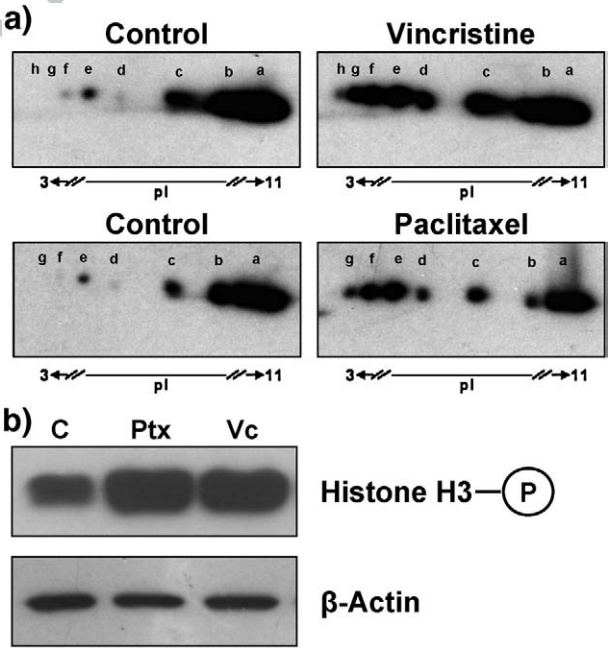

Fig. 8 – Effects of vincristine and paclitaxel over p54^{nrb} in HEK 293 cells. Cells were treated with vehicle (control), 1 μM vincristine or 1 μM paclitaxel for 24 h. a) Cell extracts were subjected to a 2D–PAGE followed by a Western blot using antibodies against p54^{nrb}. b) Cell extracts were analysed by Western blot analysis using antibodies against phosphorylated histone H3 and β-actin as loading control.

Please cite this article as: Casado P, et al, Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54^{nrb}, an event linked to G2/M arrest, J. Prot. (2008), doi:10.1016/j.jprot.2008.09.001

3.8. *p54^{nrb} phosphorylation occurs in cells other than HeLa*

The MIA-induced phosphorylation of p54^{nrb} also occurs in cell lines other than HeLa. We have studied this modification in HEK 293 cells. For this purpose, cells were treated with vehicle (control), 1 μM vincristine or 1 μM paclitaxel for 24 h. Then cell extracts were subjected to 2D–PAGE followed by Western blot analysis. This analysis showed that in HEK 293 cells, vincristine and paclitaxel induced the phosphorylation of p54^{nrb} (Fig. 8a). In addition, a Western blot analysis using antibodies against phospho-histone H3 indicated that MIAs also cause M arrest in this cell line (Fig. 8b). The KSP inhibitor STLC also induced the phosphorylation of this nuclear factor (see Fig. 2 of supplementary material).

4. Discussion

VAs and taxanes are the most used and effective drugs in cancer treatment [6]. Nevertheless, the development of drug resistance by the tumoral cells and the severe secondary effects that they cause, are problems that have not been resolved yet [10]. In order to overcome this unwanted effects, new drugs, such as KSP inhibitors, are being developed [10].

MIAs and KSP inhibitors cause G2/M arrest and cell death because they inhibit the separation of sister chromatids during mitosis [2,10]. However, many molecular effects of these drugs are still unknown. The determination of these molecular actions is of great interest since they may be related to secondary effects and resistance development.

In the last few years, the development of proteomic techniques has made 2D–PAGE followed by MS analysis a powerful tool for the analysis of complex protein mixtures. This methodology has been recently used to investigate proteins that are regulated by chemotherapy agents [23,24].

In this report, we use this technology to identify several forms of the nuclear factor p54^{nrb} that are detected after vincristine treatment. The incubation of cell extracts from drug treated cells with λ-phosphatase determined that all these forms are phosphorylated. Furthermore, we observed that all MIAs tested induce mitotic arrest and cell death while triggering p54^{nrb} phosphorylation in HeLa and HEK 293 cells. The KSP inhibitor STLC, also induces the phosphorylation of this nuclear factor. On the other hand, the drug cisplatin, which induces cell death but not M arrest, does not induce this modification. Furthermore, when cells are arrested at the beginning of S phase by treatment with the DNA polymerase inhibitor aphidicolin, MIAs are unable to induce G2/M arrest and to trigger the phosphorylation of this nuclear factor. This clearly indicates that the p54^{nrb} phosphorylation induced by these agents occurs during the G2/M phase. Moreover, the treatment with the CDK inhibitor roscovitine downregulates the p54^{nrb} forms that are phosphorylated after MIA treatment. These results indicate that CDK activity is needed for MIA-induced phosphorylation of p54^{nrb}. Roscovitine is a CDK inhibitor that blocks CDK1 and CDK2 with the same specificity and CDK5 to a less extent. In our assay, when roscovitine is added, HeLa cells are already arrested in M phase by a previous 24 h pretreatment with paclitaxel. Thus, during roscovitine exposure, the phosphorylated p54^{nrb} is dephosphorylated by phosphatases in the absence of CDK activity. Since it is considered that CDK1 is the main active CDK during this phase of the cell cycle [25], we suggest that CDK1 is responsible for p54^{nrb} phosphorylation. This agrees with other data previously published. Thus, the MIAs used here have been shown to activate CDK1 [26]. It has been described that p54^{nrb} can be phosphorylated at threonine 450 [27], a position located at a motif targeted by the mitotic kinase CDK1 [28]. It has been determined that p54^{nrb} is phosphorylated during mitosis by CDK1 [29]. Since the drugs used in this work induce mitotic arrest, it is likely that this kinase is responsible for the phosphorylation described in this work. We have tried to find the peptides containing threonine 450 in our mass spectrometry analysis. However we did not find neither the phosphorylated peptide in spot 1 nor the unphosphorylated one in spot 2 (Fig. 1a).

During cell division there is a general reduction of mRNA levels. However, there are subset of genes (many of them closely related to the mitotic process) whose mRNAs are increased [30]. The protein p54^{nrb} is a nuclear factor that participates in many cellular processes, such as transcription and splicing. Thus, it is conceivable that the phosphorylation of this protein is required for these changes in mRNA levels to occur. Further research is required to establish whether this phosphorylation is in the basis of the mitotic process itself, it is a consequence of MIA action or, alternatively, of whether it is a mechanism of cell resistance to the drugs.

In summary then, we report that the MIAs vincristine, vinblastine, paclitaxel and docetaxel, as well as the KSP inhibitor STLC, induce the phosphorylation of the nuclear factor p54^{nrb}, while the DNA damaging agent cisplatin does not cause this modification and that this phosphorylation is associated to the G2/M arrest induced by these drugs.

Acknowledgments

We thank Vanessa Díaz-Huerta and María Fernández for help with MS analysis and Ana Salas for help with flow cytometry analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2008.09.001.

REFERENCES

[1] Jordan MA, Wilson L. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. Curr Opin Cell Biol 1998;10:123–30.
[2] Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. Nat Rev Cancer 2004;4:253–65.
[3] Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T, et al. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J Biol Chem 1998;273:4928–36.

Please cite this article as: Casado P, et al, Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54^{nrb}, an event linked to G2/M arrest, J. Prot. (2008), doi:10.1016/j.jprot.2008.09.001

[4] Ruvolo PP, Deng X, May WS. Phosphorylation of Bcl2 and regulation of apoptosis. Leukemia 2001;15:515–22.
[5] Wahl AF, Donaldson KL, Fairchild C, Lee FY, Foster SA, Demers GW, et al. Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis. Nat Med 1996;2:72–9.
[6] Fojo T. Can mutations in gamma-actin modulate the toxicity of microtubule targeting agents? J Natl Cancer Inst 2006;98:1345–7.
[7] Duflos A, Kruczynski A, Barret JM. Novel aspects of natural and modified vinca alkaloids. Curr Med Chem Anticancer Agents 2002;2:55–70.
[8] Gigant B, Wang C, Ravelli RB, Roussi F, Steinmetz MO, Curmi PA, et al. Structural basis for the regulation of tubulin by vinblastine. Nature 2005;435:519–22.
[9] Blangy A, Arnaud L, Nigg EA. Phosphorylation by p34cdc2 protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150. J Biol Chem 1997;272:19418–24.
[10] Jackson JR, Patrick DR, Dar MM, Huang PS. Targeted anti-mitotic therapies: can we improve on tubulin agents? Nat Rev Cancer 2007;7:107–17.
[11] Shav-Tal Y, Zipori D. PSF and p54(nrb)/NonO—multi-functional nuclear proteins. FEBS Lett 2002;531:109–14.
[12] Kameoka S, Duque P, Konarska MM. p54(nrb) associates with the 5' splice site within large transcription/splicing complexes. Embo J 2004;23:1782–91.
[13] Zhang Z, Carmichael GG. The fate of dsRNA in the nucleus: a p54(nrb)-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs. Cell 2001;106:465–75.
[14] Straub T, Knudsen BR, Boege F. PSF/p54(nrb) stimulates "jumping" of DNA topoisomerase I between separate DNA helices. Biochemistry 2000;39:7552–8.
[15] Bladen CL, Udayakumar D, Takeda Y, Dynan WS. Identification of the polypyrimidine tract binding protein-associated splicing factor.p54(nrb) complex as a candidate DNA double-strand break rejoining factor. J Biol Chem 2005;280:5205–10.
[16] Takahashi N, Tochimoto N, Ohmori SY, Mamada H, Itoh M, Inamori M, et al. Systematic screening for genes specifically expressed in the anterior neuroectoderm during early *Xenopus* development. Int J Dev Biol 2005;49:939–51.
[17] Yang Z, Sui Y, Xiong S, Liour SS, Phillips AC, Ko L. Switched alternative splicing of oncogene CoAA during embryonal carcinoma stem cell differentiation. Nucleic Acids Res 2007;35:1919–32.
[18] Pavao M, Huang YH, Hafer LJ, Moreland RB, Traish AM. Immunodetection of nmt55/p54nrb isoforms in human breast cancer. BMC Cancer 2001;1:15.
[19] Casado P, Zuazua-Villar P, del Valle E, Martinez-Campa C, Lazo PS, Ramos S. Vincristine regulates the phosphorylation of the antiapoptotic protein HSP27 in breast cancer cells. Cancer Lett 2007;247:273–82.
[20] Chalkley RJ, Baker PR, Huang L, Hansen KC, Allen NP, Rexach M, et al. Comprehensive analysis of a multidimensional liquid chromatography mass spectrometry dataset acquired on a quadrupole selecting, quadrupole collision cell, time-of-flight mass spectrometer: II. New developments in Protein Prospector allow for reliable and comprehensive automatic analysis of large datasets. Mol Cell Proteomics 2005;4:1194–204.
[21] Otto FJ, Oldiges H, Jain UK. Flow cytometric measurement of cellular DNA content dispersion induced by mutagenic treatment. Berlin: Springer-Verlag; 1984.
[22] Schmidt F, Hustoft HK, Strozynski M, Dimmler C, Rudel T, Thiede B. Quantitative proteome analysis of cisplatin-induced apoptotic Jurkat T cells by stable isotope labeling with amino acids in cell culture, SDS–PAGE, and LC-MALDI-TOF/TOF MS. Electrophoresis 2007;28:4359–68.
[23] Verrills NM, Walsh BJ, Cobon GS, Hains PG, Kavallaris M. Proteome analysis of vinca alkaloid response and resistance in acute lymphoblastic leukemia reveals novel cytoskeletal alterations. J Biol Chem 2003;278:45082–93.
[24] Prado MA, Casado P, Zuazua-Villar P, del Valle E, Artime N, Cabal-Hierro L, et al. Phosphorylation of human eukaryotic elongation factor 1Bgamma is regulated by paclitaxel. Proteomics 2007;7:3299–304.
[25] Nigg EA. Mitotic kinases as regulators of cell division and its checkpoints. Nat Rev Mol Cell Biol 2001;2:21–32.
[26] Dangi S, Shapiro P. Cdc2-mediated inhibition of epidermal growth factor activation of the extracellular signal-regulated kinase pathway during mitosis. J Biol Chem 2005;280:24524–31.
[27] Beausoleil SA, Villen J, Gerber SA, Rush J, Gygi SP. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. Nat Biotechnol 2006;24:1285–92.
[28] Shah OJ, Ghosh S, Hunter T. Mitotic regulation of ribosomal S6 kinase 1 involves Ser/Thr, Pro phosphorylation of consensus and non-consensus sites by Cdc2. J Biol Chem 2003;278:16433–42.
[29] Proteau A, Blier S, Albert AL, Lavoie SB, Traish AM, Vincent M. The multifunctional nuclear protein p54nrb is multiphosphorylated in mitosis and interacts with the mitotic regulator Pin1. J Mol Biol 2005;346:1163–72.
[30] Whitfield ML, Sherlock G, Saldanha AJ, Murray JI, Ball CA, Alexander KE, et al. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell 2002;13:1977–2000.

Please cite this article as: Casado P, et al, Microtubule interfering agents and KSP inhibitors induce the phosphorylation of the nuclear protein p54^{nrb}, an event linked to G2/M arrest, J. Prot. (2008), doi:10.1016/j.jprot.2008.09.001