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Inhibition of the mammalian target of rapamycin (mTOR) by rapamycin increases chemosensitivity of CaSki cells to paclitaxel

L.S. Faried[a,*], *A. Faried*[b], *T. Kanuma*[a], *T. Nakazato*[a], *T. Tamura*[a], *H. Kuwano*[b], *T. Minegishi*[a]

[a]*Department of Gynecology and Reproductive Medicine, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan*

[b]*Department of General Surgical Science (Surgery I), Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan*

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ABSTRACT

Paclitaxel, a potent anti-neoplastic agent, has been found to be effective against several tumours, including cervical cancer. However, the exact mechanism underlying the cytotoxic effects of pacitaxel, especially in the survival-signalling pathway, is poorly understood. The aim of this study was to investigate the molecular pathway of the cytotoxic effect of paclitaxel in human cervical cancer cell lines. Four human cervical cancer cell lines were treated for 24 h with various concentration of paclitaxel, and the sensitivity was analysed by an MTT assay. The cell cycle progression and sub-G1 population were analysed by flow cytometry. Apoptosis was further measured by DNA fragmentation and microscope examination. The protein expression was determined by Western blot analysis. Our results showed that HeLa cells demonstrated the highest sensitivity to paclitaxel, whereas CaSki cells showed the lowest. In cervical cancer cells, paclitaxel induced apoptosis through an intrinsic pathway with prior G2/M arrest. In addition, we showed that paclitaxel downregulated the phosphorylation of Akt in both HeLa and CaSki cells. Interestingly, in CaSki cells, which were more suggestive of a resistant phenotype, paclitaxel induced the activation of mTOR as a downstream target of Akt. Pre-treatment with rapamycin inhibited activation of mTOR signalling and significantly enhanced the sensitivity of CaSki cells to paclitaxel by increasing apoptotic cell death. This effect was mediated, at least partly, through caspase activation. Overall, paclitaxel exerts its anti-tumour effects on cervical cancer cells by inducing apoptosis through intrinsic pathway, and rapamycin targeted to mTOR can sensitise paclitaxel-resistant cervical cancer cells.

1. Introduction

Carcinoma of the cervix, the second most common neoplasm in women worldwide, constitutes 12% of all female cancer and causes 250,000 deaths per annum.[1] The incidence of cervical cancer is the highest in developing countries and is the leading cause of female cancer mortality.[2] Patients with advanced, persistent, or recurrent squamous cell carcinoma of the cervix, which cannot be treated with surgical resection or radiation therapy, have a low survival rate.[3] The need for

* *Corresponding author:* Tel.: +81 27 220 8423; fax: +81 27 220 8443.
E-mail address: leri@med.gunma-u.ac.jp (L.S. Faried).
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effective chemotherapy has yet to be defined by increasing our understanding of the molecular effect of chemotherapeutic agents in cancer cells.

Paclitaxel (Taxol), a naturally anti-neoplastic agent, is widely used in the treatment of a variety of solid tumours.[4–6] Trials have been performed to expand the list of tumour diseases that can be treated with paclitaxel, including cervical cancer.[5,7–9] At the cellular level, paclitaxel binds to β-tubulin subunits in microtubules, thus promoting the polymerization of tubulin and disrupting microtubule dynamics, leading to mitotic arrest and, subsequently, to apoptotic cell death.[10] Two signalling pathways for the initiation of apoptosis are well known. One is mediated by a dead receptor on the cell surface, which is called an "extrinsic pathway", and the other, the "intrinsic pathway", is mediated by mitochondria.[11,12] In both pathways, the induction of apoptosis leads to the activation of the initiator caspase: caspase-8 for the extrinsic pathway and caspase-9, which is activated at the apoptosome, for the intrinsic pathway.[13] These initiator caspases then activate caspase-3 to execute apoptotic cell death, DNA fragmentation, and chromatin condensation.[14,15] Caspase-3 and caspase-9 have been shown to cleave the 116 kDa nuclear protein poly (ADP-ribose) polymerase "PARP" into an 85 kDa apoptotic fragment.[16,17]

It is accepted that the mechanism of drug-induced apoptosis is governed not only by the upregulation of pro-apoptotic factors or tumour suppressors but also by the modulation of the survival-signalling pathways.[18] One of the important survival-signalling pathways is mediated by phosphoinositide 3-OH kinase "PI3K" and its downstream target, Akt.[19] Recent studies suggest that paclitaxel affects the activities of Akt in lung, oesophageal, ovarian and pancreatic cancer cells and that the inhibition of Akt activities enhances the cytotoxic effect of chemotherapy agents.[20–22] The mammalian target of rapamycin (mTOR) is one critical target of Akt in survival-signalling. Akt phosphorylates tuberous sclerosis complex 2, leading to the dissociation of tuberous sclerosis complexes 1 and 2. Together, tuberous sclerosis complexes 1 and 2 compose a GTPase complex for Rheb, which, in turn, activates mTOR.[23] In addition, Akt may directly activate mTOR by phosphorylating an auto-inhibitory region of mTOR.[24] The best characterised function of mTOR is the regulation of translation by phosphorylating ribosomal S6 kinase (S6K1) and the eukaryote initiation factor 4E-binding protein (4E-BP1).[25]

Although numerous reports have determined the molecular mechanism of paclitaxel, its effects on the survival-signalling pathway, especially, in cervical cancer cells, are not well understood. In this study we investigated the different sensitivity of human cervical cancer cell lines to paclitaxel and if it could induce apoptosis in those cells. Further, we used HeLa and CaSki cells to evaluate the possible involvement of several apoptosis regulator proteins. We also analysed the effect of paclitaxel on the expression of survival-signalling pathway proteins, such as Akt and mTOR. Additionally, LY294002 (PI3K inhibitor) or rapamycin (mTOR inhibitor) were used to determine whether pre-treatment with these inhibitors would be more effective in exerting the cytotoxic effects of paclitaxel in human cervical cancer cell lines.

2. Materials and methods

2.1. Cell lines and culture conditions

Four human cervical cancer cell lines, HeLa, SiHa, ME180, and CaSki, were purchased from the American Type Culture Collection (Manassas, VA). HeLa and SiHa cells were maintained in Eagle's Minimum Essential Medium (EMEM) obtained from Sigma Chemical Co. (St. Louis, MO), supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (FBS) obtained from Gibco (BRL, Grand Island, NY). ME180 cells were maintained in McCoy5a Modified Medium (Gibco) supplemented with 10% FBS. CaSki cells were maintained in RPMI-1640 medium (Gibco) supplemented with HEPES (Sigma) and 10% FBS.

2.2. Drugs and cell treatment

Paclitaxel was purchased from Wako Pure Chemicals (Osaka, Japan) and received as sterile lyophilized powder. A stock solution of 5 mg/ml was made in dimethylsulfoxide (DMSO) and stored at 4 °C; further dilutions were made in culture media to obtain the desired concentrations when the cells reached approximately 80% confluency. The final concentration of DMSO did not exceed 0.08%, a concentration that does not alter the growth or survival properties of any cell types. We used a paclitaxel peak plasma concentration (ppc) of 5 μM = 4270 ng/ml.[5] LY294002 and rapamycin were purchased from Calbiochem (San Diego, CA). Cells were exposed for 24 h to 5 μM paclitaxel. For LY294002 (25 μM) or rapamycin (100 nm), cells were pre-incubated with these inhibitors for 6 h prior to paclitaxel treatment.

2.3. Drug sensitivity assay

Cell proliferation analysis was performed on human cervical cancer cell lines in the presence of various concentrations of drugs by a colorimetric methyl thiazolyl tetrazolium (MTT) assay as described previously.[26] Briefly, exponentially growing cells ($\sim 2 \times 10^4$ cells/well) were plated in 96-well plates. After an overnight culture, the medium was changed to a fresh medium with different concentrations of drugs. At the end of various treatments, 10 μl of a cell-counting solution (WST-8, Dojindo Labs, Tokyo, Japan) was added. After dissolving the crystals with 1 N HCl-isopropanol, the absorbance was measured at a wavelength of 450 nm using a microtiter plate reader (Beckton Dickinson, Franklin Lakes, NJ). A value of 100% was assigned to untreated control, and the concentration of drug that reduce the number of viable cells to 50% after 24 h of exposure (IC_{50}) was derived from cell survival plots. All experiments were performed in triplicate.

2.4. Light microscopy examination

Cells were cultured in a 6-cm plate and treated with drugs as described. Morphological changes were examined at the times indicated and photographed using a regular phase-contrast microscope.

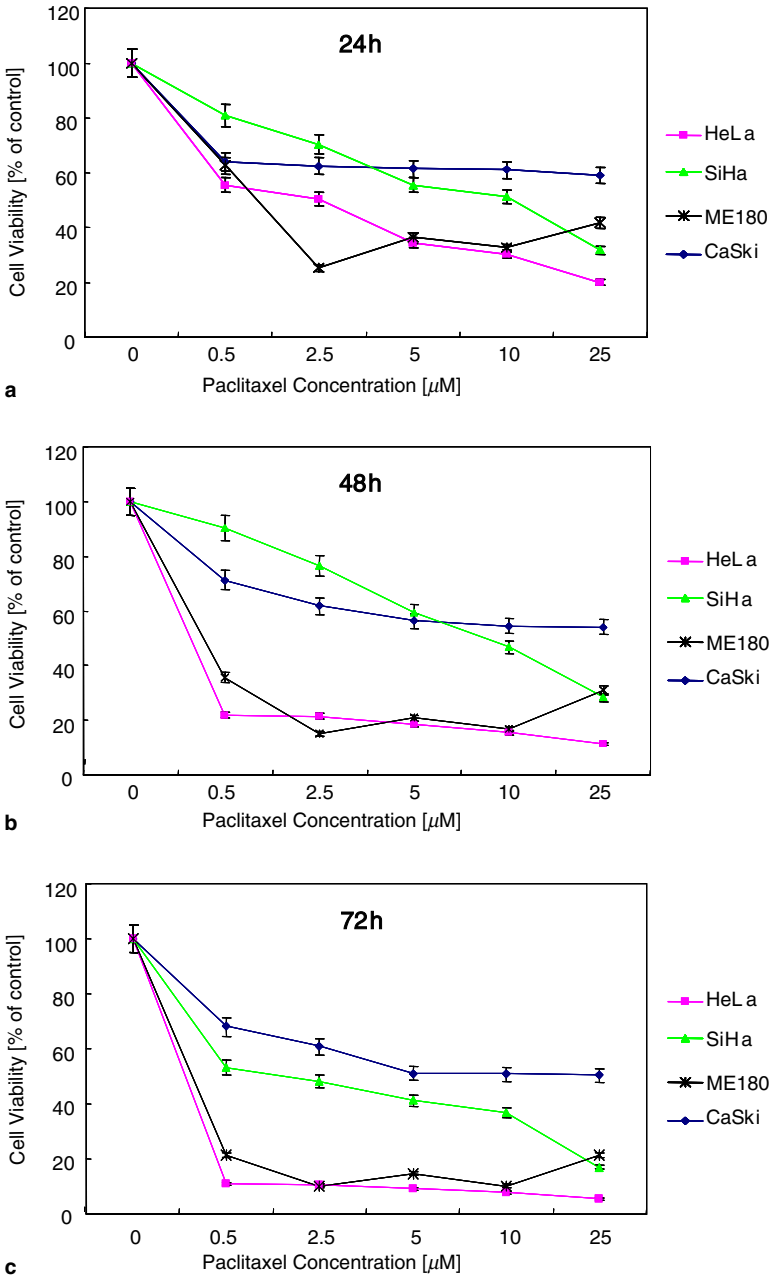

Fig. 1 – The effect of paclitaxel on the growth inhibition of cervical cancer cells was compared using MTT assay. Concentration- and time-related inhibition of HeLa, SiHa, ME-180, and CaSki cells growth at (a) 24 h, (b) 48 h, and (c) 72 h were shown. The values represent means ± SD from three independent experiments.

2.5. *Detection of sub-G1 DNA content by propidium iodide staining*

The fraction of cells containing sub-G1 DNA content has been shown to correlate with apoptotic cell death[27] and was assessed by flow cytometry analysis as described previously.[26] Briefly, at the end of each time point, both detached and attached cells were collected and washed twice with ice-cold phosphate buffer saline (PBS). Cells were then stained with a propidium iodide (PI) solution and incubated at 37 °C for 30 min with RNAse. The DNA content was analysed using the FACScan Coulter Epics XL Flow Cytometer (Coulter Corp. USA). The distribution of cells in the different phases of the cell cycle was analysed from the DNA-histograms using EXPO™ 32 software.

2.6. *DNA extraction and gel electrophoresis*

DNA isolation was performed as described previously.[26] Briefly, cells were harvested at the time indicated, washed with cold PBS and pelleted by centrifugation at 300*g* for 10 min. Cell pellets were resuspended in lysis buffer (5 mM

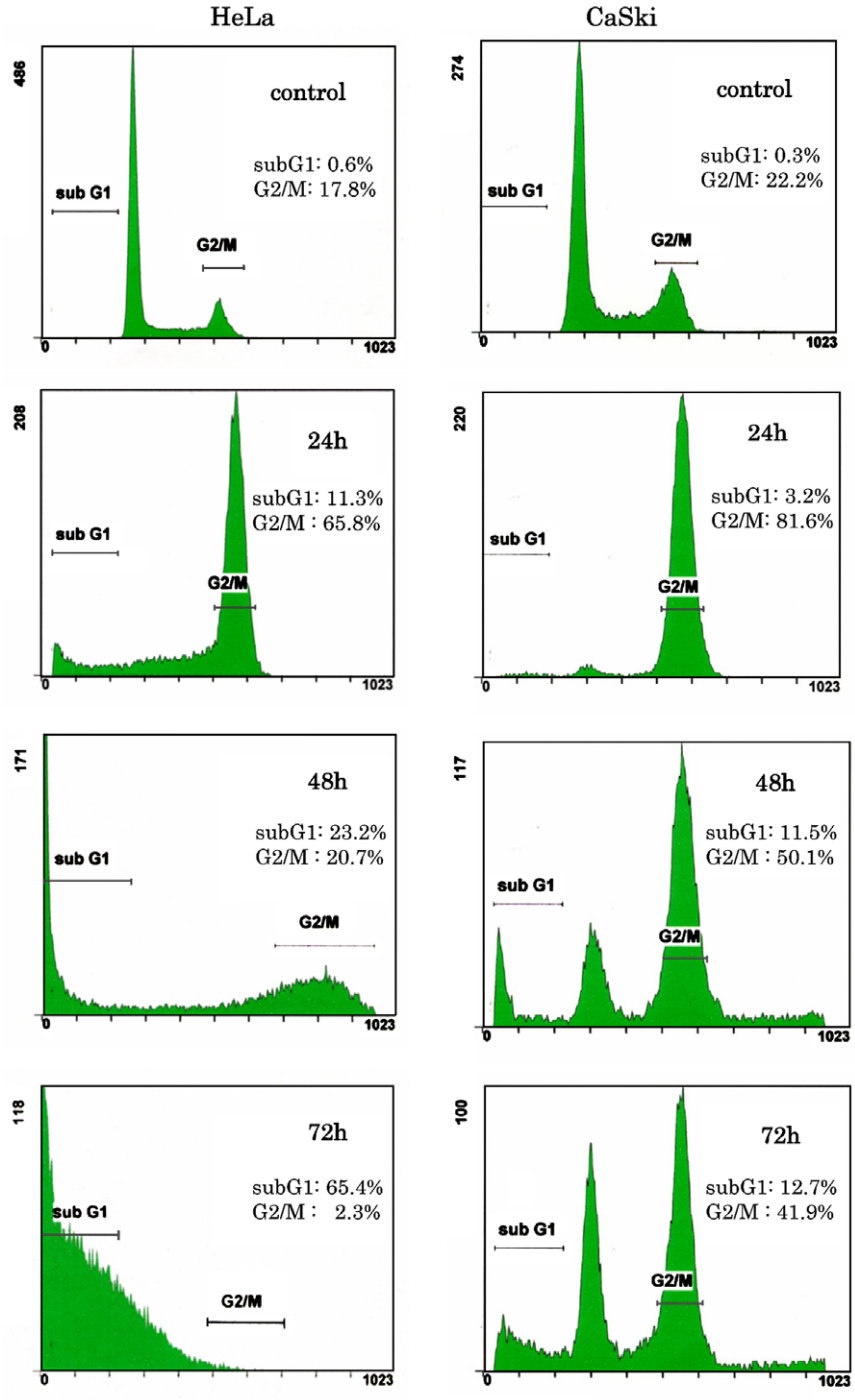

Fig. 2 – The effects of paclitaxel on the cell cycle. Cells were treated with 5 μM of paclitaxel for 24 h and harvested at the indicated times. PI-labelled cells were analysed with flow cytometry, demonstrating the accumulation of the cells in G2/M phase and the development of a sub-G1 population. The distribution of cells in the sub-G1 and G2/M phase of the cell cycle is indicated within each plot. The results are representative of three independent experiments.

Tris at pH 7.5, 0.5% Triton X-100, 20 mM EDTA) and the DNA was extracted with phenol–chloroform and precipitated with 2 volume of ethanol at −20 °C for 24 h. The DNA was then resuspended in TE buffer (10 mM Tris at pH 7.5, 10 mM EDTA) and quantified by absorbance at 260 nm (Beckman DU 640, USA). DNA (5 μg) was applied to 2% agarose gel and electrophoresed at 50 V for 45 min and the gels were stained with ethidium bromide. The DNA bands were visualised using UV transluminator and photographed with Polaroid film.

2.7. *Western blot analysis*

Western blot analysis was done as described previously.[26] Cells were harvested at the time indicated and prepared in a

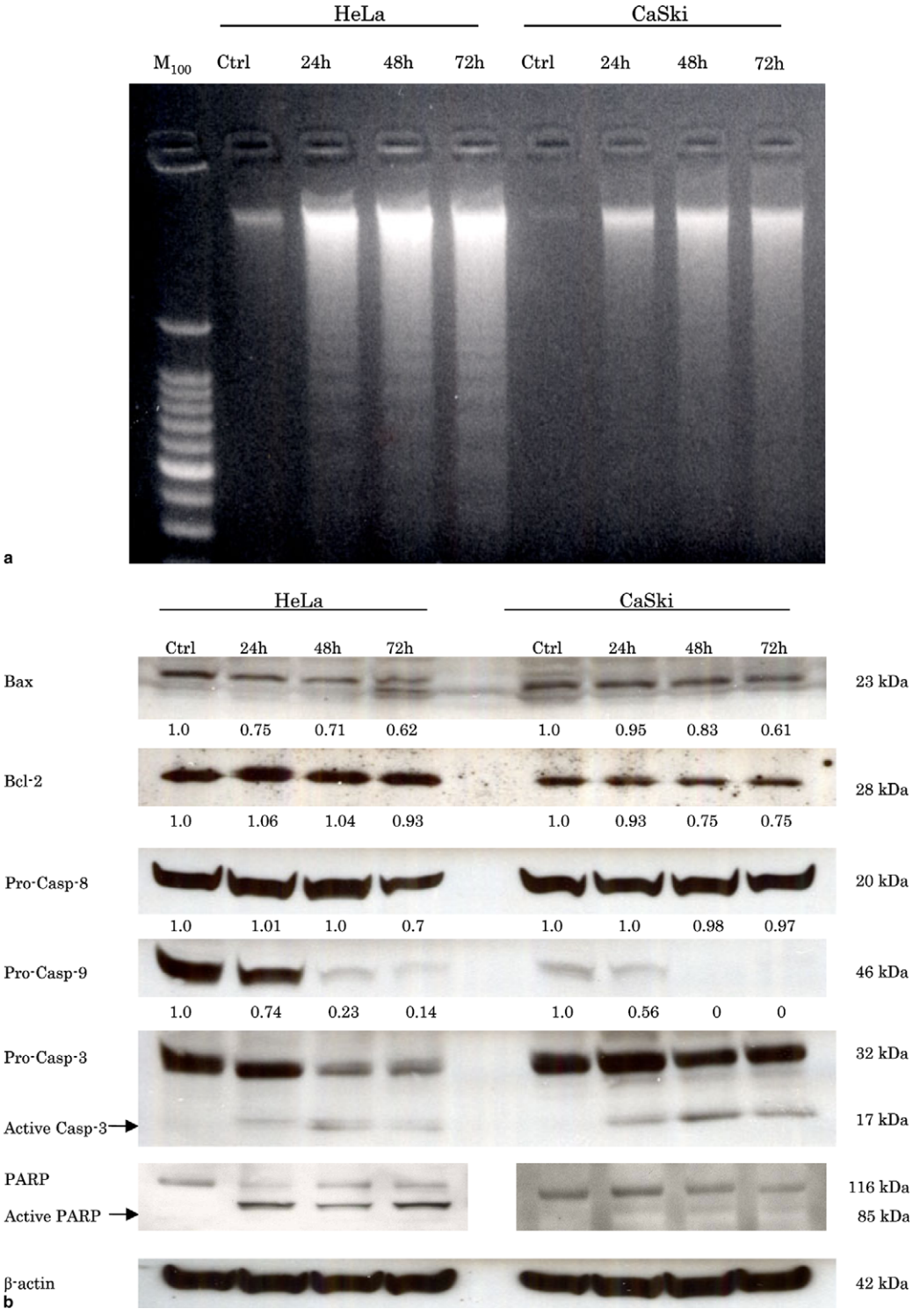

Fig. 3 – Paclitaxel induced apoptosis in human cervical cancer cells. (a) DNA fragmentation assay of cervical cancer cells. Cells were treated with 5 μM of paclitaxel for 24 h and then harvested at 24, 48, and 72 h after treatment. DNA was extracted and analysed by electrophoresis in 2% agarose gel containing ethidium bromide. Experiments were repeated twice with similar results. M: Marker DNA 100 base pairs. (b) Western blot analysis of Bax, Bcl-2, pro-caspase-8, pro-caspase-9, caspase-3, and PARP expression in HeLa and CaSki cells treated with paclitaxel for 24 h and harvested for the times indicated. The values underneath the bands represent the densitometric estimation of the density of the bands. The expression of β-actin served as loading control. Experiments were repeated twice with similar results.

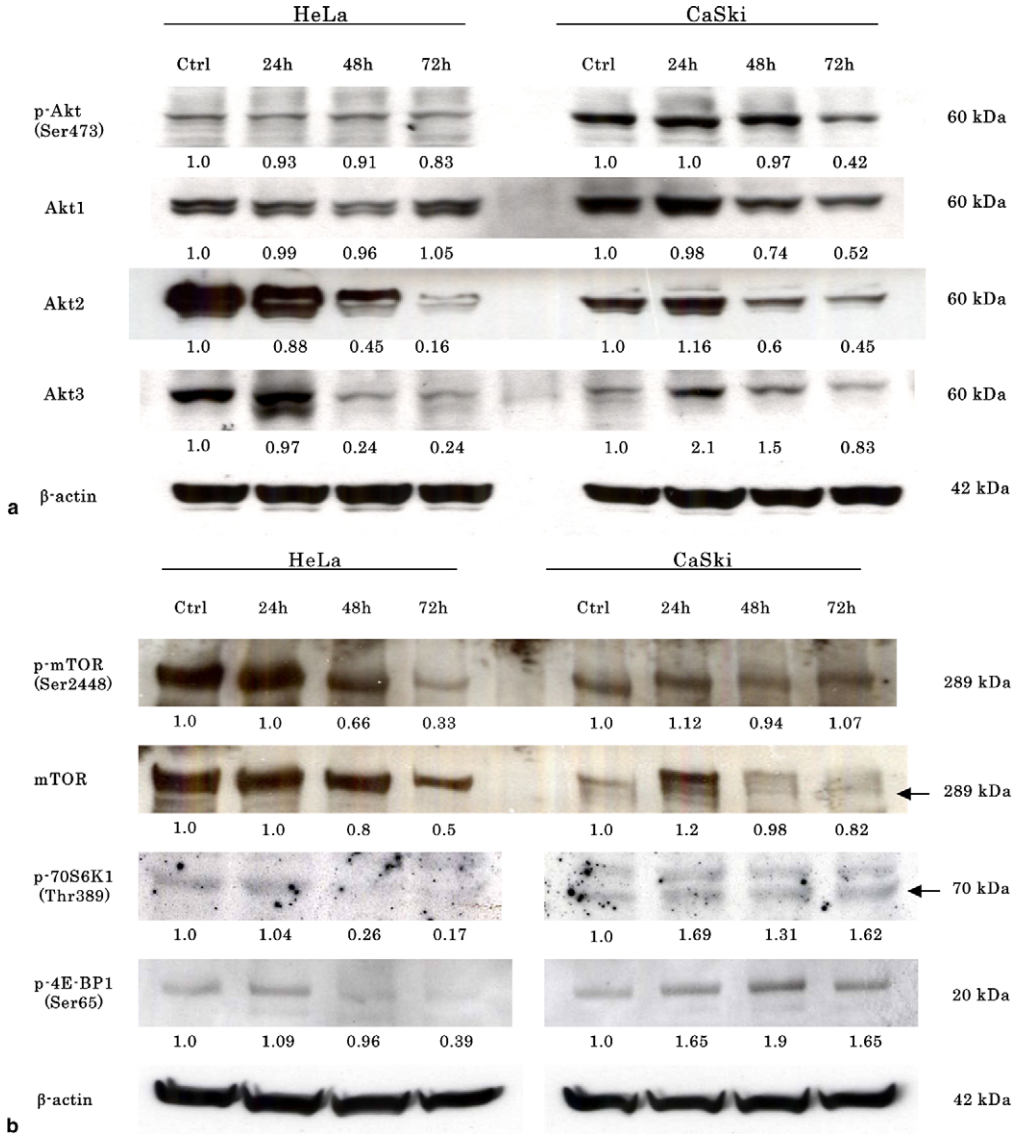

Fig. 4 – Paclitaxel effect on the expression of (a) Akt and (b) mTOR signalling pathway was assessed by Western blotting. The values underneath the bands represent the densitometric estimation of the band density. The expression of β-actin served as loading control. Experiments were repeated twice with similar results.

buffer (20 mM Tris–HCl, pH 7.6, 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1% aprotinin, 1 mM phenylmethylsulfonylfluoride, and 1 mM sodium vanadate). The protein concentration of the sample was determined by a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Equal amounts of protein (40 μg) were subjected to a 5–20% Tris-tricine Ready Gel (Bio-Rad, Tokyo, Japan) and then transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membrane was blocked with Tris-buffered saline/0.1% Tween 20 with 5% nonfat dry milk and incubated in primary antibodies against Bax, Bcl2, caspase-8, -9, -3, and NFκB (Santa Cruz); p-Akt (Ser473), Akt1, Akt2, Akt3, p-mTOR (ser2448), mTOR, p-S6K1 (Thr389), p-4E-BP1 (Cell Signalling); XIAP (PharMingen) at 4 °C overnight. The bands were visualised by a chemiluminescence system (Amersham). The expression of β-actin (Sigma) served as loading control. The density of the bands was quantified using Quantity One (BioRad).

2.8. *Statistical analysis*

Quantitative experiments were analysed by Student's *t* test. *P*-values resulting from the use of two-sided tests and were considered significant when $P < 0.05$.

3. Results

3.1. *Sensitivity of human cervical cancer cells to paclitaxel*

To investigate the sensitivity of human cervical cancer cells to paclitaxel, the cells were treated for 24 h in a medium containing varying concentrations of paclitaxel. Exposure to paclitaxel (0.5–25 μM) produced a dose- and time-dependent reduction in cell growth in HeLa, ME180, SiHa, and CaSki cells (Fig. 1a–c). HeLa cells demonstrated the highest sensitivity among all cell types; at 24 h, they showed decreases of 44.6% and 80.1% in cell

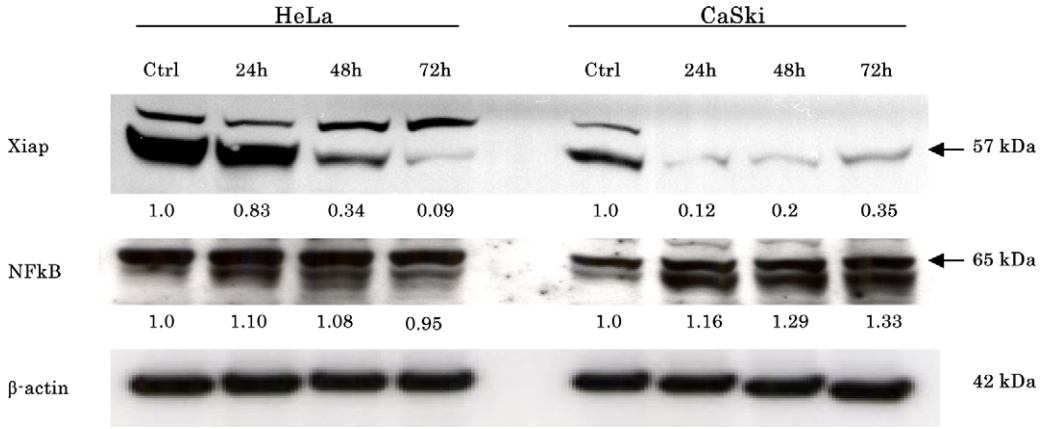

Fig. 5 – The expression of Xiap and NFκB protein, cells were treated with paclitaxel for 24 h and harvested at the indicated times. The values underneath the bands represent the densitometric estimation of the bands density. The expression of β-actin served as loading control. Experiments were repeated twice with similar results.

viability at 0.5 and 25 μM, respectively, whereas CaSki cells demonstrated the lowest sensitivity, with decreases of 36.1% and 41.1% in cell viability at 0.5 and 25 μM, respectively (Fig. 1a). The inhibition concentration 50 (IC_{50}) values were 3.5, 3.75, and 8.75 μM for HeLa, ME180, and SiHa cells, respectively; on the other hand, CaSki cells did not reach the IC_{50} values until the highest dose had been given. Based on these results, we used HeLa and CaSki cells for further experiments.

3.2. *Paclitaxel enhanced the sub-G1 population in cervical cancer cells*

To examine the cytotoxic effects of paclitaxel, we performed flow cytometry analysis to measure the presence of the sub-G1 population, which refers to apoptosis cells.[27] As shown in Fig. 2, the incubation of HeLa and CaSki cells with 5 μM paclitaxel resulted in mitotic blockage in the G2/M phase as early as 24 h and reached the highest level (65.8–81.6%), followed by an increased sub-G1 population at 48 and 72 h. These results suggest that the cells were undergoing apoptosis after prolonged mitotic blockage. In both cells examined, treatment with paclitaxel resulted in a time-dependent accumulation of the sub-G1 population. HeLa cells demonstrated a high number of cells in the sub-G1 population, with a peak occurring at 72 h (65.4%); on the other hand, CaSki cells had a much lower sub-G1 population (12.7%). Although CaSki cells demonstrated a developed increased of sub-G1 population, most of the cells remained in the G2/M phase within 72 h (41.9%). The paclitaxel effect in SiHa and ME180 cells was similar to that observed in HeLa and CaSki cells (data not shown).

3.3. *Paclitaxel induced apoptosis in human cervical cancer cells*

To clarify the mode of cell death caused by paclitaxel, we examined the effects on internucleosomal DNA fragmentation which is a classical characteristic feature of apoptosis. As shown by 2% agarose gel electrophoresis, a time-dependent DNA fragmentation response was apparent in both cell types when the cells were treated with 5 μM of paclitaxel for 24 h. However, HeLa cells exhibited a cleared and marked DNA ladder pattern as early as 24 h and increased by time course in comparisons with CaSki (Fig. 3a). These results were consistent with the results of the MTT assay and flow cytometry and imply that the cytotoxic action of paclitaxel was due to its ability to induce apoptosis.

Furthermore, we evaluated the expression of apoptosis-related proteins, namely, Bax, Bcl-2, caspase-8, caspase-9, caspase-3, and PARP, by Western blot analysis. Apoptosis can be regulated by relatives of Bax and Bcl-2 proteins, members of the Bcl-2 family of apoptosis-promoting and apoptosis-inhibitory factors.[28] As shown in Fig. 3b, that although the Bax and Bcl2 level was slightly decreased in both cell types, we have observed that paclitaxel-induced downregulation of pro-caspase-9 and activation of caspase-3, followed by cleavage its 85 kDa substrate protein, PARP. There is not much modified expression of pro-caspase-8. These results indicate that paclitaxel induced apoptosis, at least partly, through an intrinsic pathway in cervical cancer cells.

3.4. *Paclitaxel treatment altered the expression of PI3K/Akt-signalling proteins*

One of the most important survival-signalling pathways is mediated by PI3K and its downstream targets, such as Akt and mTOR.[19] It was recently reported that Akt plays an important role in determining the chemo-sensitivity of many kinds of cells.[20–22] We evaluated the expression of these survival-signalling proteins in response to paclitaxel exposure in cervical cancer cells. Fig. 4a showed that paclitaxel inhibited the activation of Akt in both HeLa and CaSki cells, as shown by the reduced expression of phosphorylated Akt (Ser473). The expression of Akt2 and Akt3 in HeLa cells was also decreased by paclitaxel within 72 h. Furthermore, although the expression of Akt2 and Akt3 in CaSki cells was increased at 24 h, paclitaxel downregulated Akt2 and Akt3 within 72 h. Paclitaxel also reduced the expression of phosphorylated mTOR as the downstream target protein of Akt in HeLa cells (Fig. 4b). On the other hand, even though the activation of Akt was decreased in CaSki cells, there is no reduction in

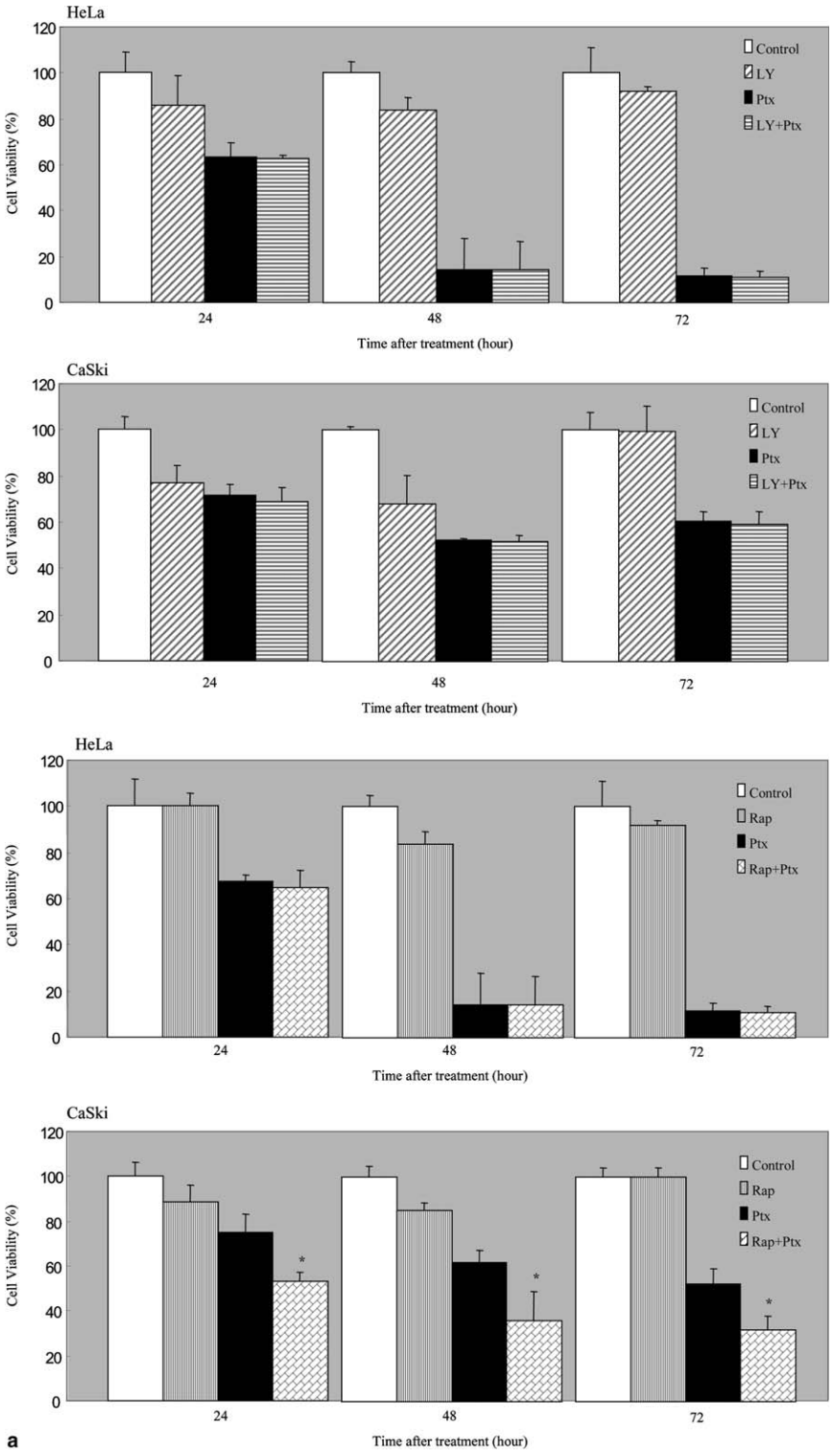

Fig. 6 – Rapamycin enhances chemo-sensitivity of cervical cancer cells to paclitaxel. Cells were treated with paclitaxel alone with or without 25 μM LY294002 or 100 nM rapamycin pre-treatment for 6 h, and harvested at the time indicated. (a) Cell growth inhibition was determined by MTT assay. The values represent means ± SD from three independent experiments (* $P < 0.05$ compare with paclitaxel-treated cells). (b) Morphological assessment in HeLa and CaSki cells in control cultures and after treatment with various drugs for 24 h using phase-contrast microscopy (200× magnification). (c) DNA fragmentation analysis, M: DNA marker 100 bp.

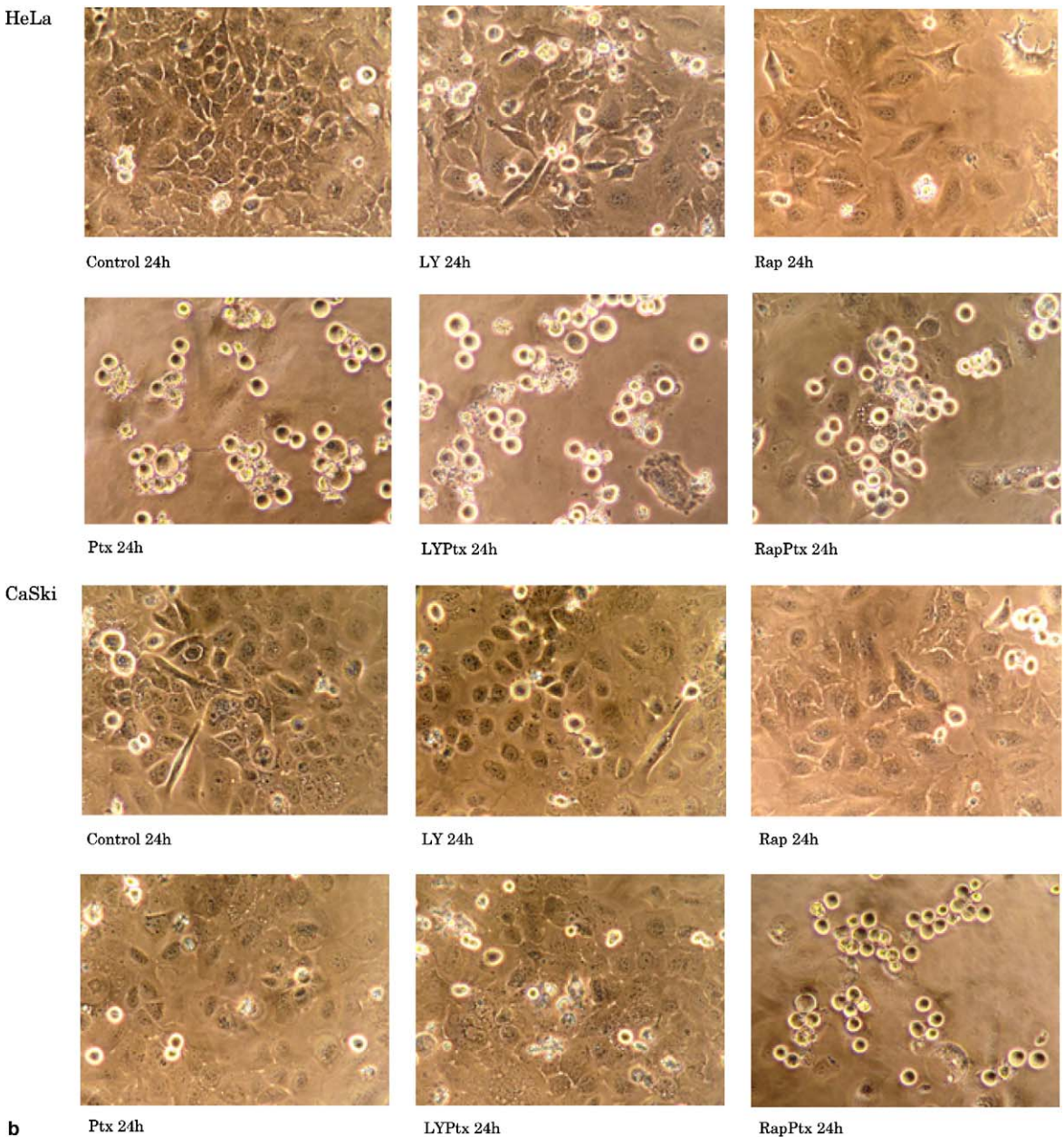

Fig. 6 – *continued*

the expression of phosphorylated mTOR after the paclitaxel treatment. In fact, paclitaxel increased the phosphorylated mTOR protein at 24 h in CaSki cells, followed by activation of its downstream proteins, S6K1 and 4E-BP1 (Fig. 4b). These results indicate that the mTOR pathway might have an important role to maintain the survival of CaSki cells in response to paclitaxel.

3.5. *Expression and modulation of other apoptosis-related proteins by paclitaxel*

The expression of two other key apoptosis regulator proteins, including NFκB and XIAP, was analysed. It has been reported that NFκB is activated by various chemotherapeutic agents, including paclitaxel.[29] NFκB activity confers resistance to paclitaxel in prostate cancer cells.[30] Our results showed that the levels of NFκB were slightly increased in CaSki and there was no modified expression in HeLa cells (Fig. 5).

The inhibitors of apoptosis proteins (IAPs) are a family of intracellular anti-apoptotic proteins that play a key role in cell survival by modulating death-signalling pathways at the post-mitochondrial level.[31] Among human IAPs, Xiap is a potent inhibitor of caspases and apoptosis. It has been shown that Xiap is a direct inhibitor of caspase-3 and caspase-9.[32] In this study, the paclitaxel treatment had significantly downregulated Xiap expression in both cell types (Fig. 5), demonstrating that Xiap plays a significant role in paclitaxel-induced apoptosis.

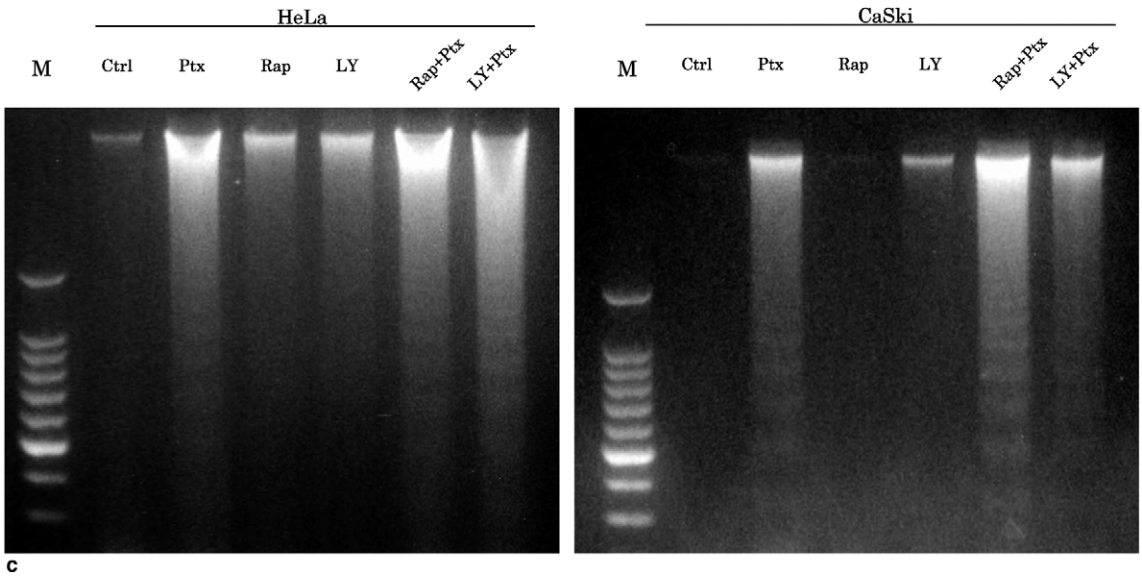

Fig. 6 – *continued*

3.6. *Inhibition of mTOR by rapamycin increases the sensitivity of paclitaxel-induced apoptosis in CaSki cells*

To confirm whether the Akt/mTOR pathway played a role in response to paclitaxel, we used LY294002 and rapamycin as pre-treatment to paclitaxel. As shown in Fig. 6a, pre-treatment with LY294002 did not significantly enhance the cell proliferation inhibition in either HeLa or CaSki cells. On the other hand, pre-treatment with rapamycin significantly enhanced the chemo-sensitivity of CaSki cells to paclitaxel. To further evaluate the effect of pre-treatment with these inhibitor agents on apoptosis, we analysed morphological changes using phase-contrast microscopy. The results showed that pre-treatment with rapamycin induced apoptosis in most of CaSki cells, which appeared as bright, rounded, and detached cells from the dish (Fig. 6b). Next, we analysed the effect of pre-treatment with these inhibitor agents on paclitaxel treatment by a DNA fragmentation assay. LY294002 or rapamycin alone did not induce DNA ladder formation in either cell types, and pre-treatment with rapamycin enhanced the formation of the DNA ladder in CaSki cells more than paclitaxel alone did (Fig. 6c). These results indicate that pre-treatment with rapamycin sensitised CaSki cells to paclitaxel-mediated apoptosis.

3.7. *Inhibition of mTOR activation by rapamycin correlates with apoptosis in CaSki cells*

We investigated the expression of activated Akt and mTOR after pre-treatment with LY294002 or rapamycin. As shown in Fig. 7a, treatment of LY294002 alone inhibited the activation of Akt and mTOR signalling in both cell types, and pre-treatment with rapamycin in CaSki cells reduced the activation of mTOR significantly when compared with paclitaxel alone. Rapamycin inhibits mTOR activation, as shown by the downregulation of phosphorylated S6K1 and 4E-BP1 as the effector downstream of mTOR. These results suggest that the downregulation of mTOR activity is necessary for paclitaxel-induced chemo-sensitivity in CaSki cells to trigger apoptotic cell death.

To evaluate whether the reduction of activated mTOR after pre-treatment with rapamycin was correlated with apoptosis in CaSki cells, we analysed its effect on Xiap, caspase-3, and PARP protein expression. As shown in Fig. 7b, treatment with rapamycin prior to paclitaxel, but not with either agent alone, increased the expression of cleaved form of caspase-3 and PARP, which are essential for apoptosis. These results indicate that the reduced activation of mTOR in CaSki cells was associated with increased apoptosis.

4. Discussion

Paclitaxel, one of the broadest-spectrum anticancer agents, is currently used in the treatment of many types of advanced cancer, including carcinoma of the cervix.[7–9] Until now, the prognosis of patients with advanced, persistent, or recurrent squamous cell carcinoma of the cervix has been poor.[3] Resistance to chemotherapy is the most frequent obstacle to effective treatment. Although the molecular mechanisms of paclitaxel in the mediation of cell death are well characterised, its effects on survival signalling remain unclear.

In this study, we determined the sensitivity of human cervical cancer cells to paclitaxel, and the results from the MTT assay showed that HeLa cells had the highest sensitivity among all cell types, whereas CaSki cells were the most resistant. Furthermore, we examined whether paclitaxel induced apoptosis in human cervical cancer cells. Through flow cytometry analysis and a DNA fragmentation assay, we determined that 5 μM of paclitaxel induced apoptosis in human cervical cancer cells, as shown by an increased sub-G1 population and the appearance of the DNA ladder pattern. Consistent with the results from the MTT assay, HeLa cells demonstrated the highest number of sub-G1 population and

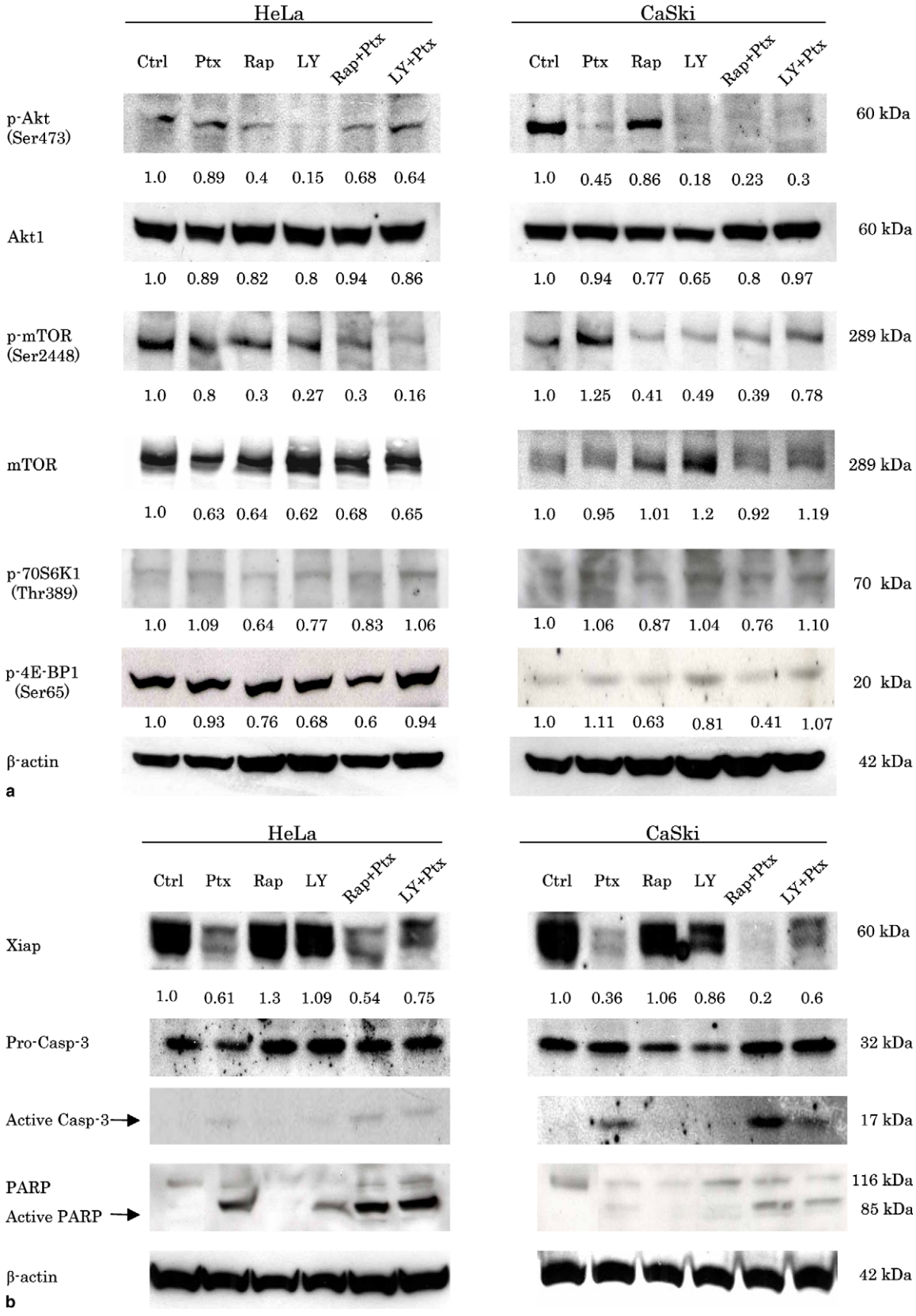

Fig. 7 – Effect of pre-treatment with rapamycin was analysed by Western blotting. (a) Rapamycin downregulated activation of mTOR by paclitaxel. (b) Rapamycin enhanced the cleavage of pro-caspase-3 and PARP to its active fragments induced by paclitaxel. The values underneath the bands represent the densitometric estimation of the bands density. The β-actin served as loading control. Experiments were repeated twice with similar results.

cleared internucleosomal DNA fragmentation. We also found that apoptosis occurred after a prolonged mitotic block in human cervical cancer cells. These results agreed with those in previous studies, which demonstrated that paclitaxel-induced apoptosis is cell cycle-dependent.[26,33]

To determine the molecular mechanism that mediates the cytotoxic effect of paclitaxel in human cervical cancer cells, we performed Western blot analysis. In this study, we demonstrated that paclitaxel only activated caspase-9 and caspase-3, without affecting the level of caspase-8 protein. These results indicate that, in human cervical cancer cell lines, apoptosis induction by paclitaxel might take place through an intrinsic pathway in inducing the caspase cascade.

It was recently reported that paclitaxel activates a serine/threonin protein kinase Akt, which is the downstream target of PI3K.[21,34] In contrast, our results demonstrated that paclitaxel inhibits the activation of Akt, as shown by the downregulation of phosphorylated Akt at serine 473. Paclitaxel downregulated Akt2 and Akt3 isoforms in both cell types within 72 h. Akt has the main role in promoting cell survival and is frequently associated with chemo-resistance to cytotoxic drugs. The elevation level of phosphorylated Akt was reported to be involved in the mechanism of chemo-resistance.[35] To our surprise, however, the downregulation of Akt did not influence the sensitivity of CaSki cells to paclitaxel-induced apoptosis. Therefore, we analysed the expression of the mTOR protein as a downstream target of Akt, and we found that, in CaSki cells, which were more suggestive of a resistant phenotype, there was no reduction in mTOR levels after paclitaxel treatment. In fact, there was increased activation of mTOR at 24 h. It is tempting to speculate that mTOR might have an important role in promoting the survival of CaSki cells.

To elucidate this phenomenon, we conducted experiments using LY294002 or rapamycin. We observed that the inhibition of Akt activation using LY294002 did not significantly enhance the sensitivity to paclitaxel in CaSki cells. One possible explanation for these results is that LY294002 could not inhibit mTOR activation as downstream of Akt might be that activation of mTOR is independent of Akt activation. The upstream effectors that activate mTOR have been extensively investigated in the past few years.[36] Several studies have reported that Akt can activate mTOR either by direct phosphorylation or via inactivation of its repressor, TSC2.[23,24] However, though serine 2448 is a consensus phosphorylation site for Akt, recent studies have revealed that serine 2448 can be phosphorylated even when PI3K/Akt is not activated.[37,38] More recent studies have reported that mTOR phosphorylation is regulated by a phospholipase Cγ-controlled calcium-signalling pathway, independent of Akt activation.[39,40]

Rapamycin has been found to inhibit serine/threonin kinase mTOR by binding to the immunophilin FK506-binding protein 12 (FKBP12). The inhibition of mTOR kinase leads to dephosphorylation of its two major downstream signalling components, p70 S6 kinase (S6K1) and 4E-BP1.[41] It was recently reported that, in vitro, rapamycin has a synergistic effect with paclitaxel, carboplatin, and vinorelbine and that a combination of rapamycin with paclitaxel leads to a significant reduction in tumour growth in vivo.[42] Furthermore, we used rapamycin to inhibit mTOR activation in both Hela and CaSki cells. As expected, we found that the inhibition of mTOR significantly enhanced the sensitivity of CaSki cells to paclitaxel. Analysis of the morphology of cells under light-microscopy and a DNA fragmentation assay strengthened our findings that the inhibition of mTOR effectively increased paclitaxel-induced apoptosis. To confirm apoptosis in response to pre-treatment with rapamycin, we evaluated caspase-3 and PARP cleavage by Western blot analysis. It was noted that the activation of caspase-3 and its 85 kDa substrate protein PARP by paclitaxel was significantly upregulated after pre-treatment with rapamycin in CaSki cells. To determine the mechanism of rapamycin-enhanced paclitaxel-induced apoptosis, we next analysed its effect on the expression of the downstream proteins of mTOR: S6K1 and 4E-BP1. S6K1 has been found to phosphorylate and inactivate the pro-apoptotic molecule Bad, a process inhibited by rapamycin.[43] When phosphorylated, the other downstream target of mTOR, 4E-BP1, releases the cap-binding protein eIF4E from a functionally inactive complex, enabling it to activate translation.[44] 4E-BP1 itself undergoes caspase-dependent cleavage in cells undergoing apoptosis, leading to an NH2-terminally truncated polypeptide that fails to become highly phosphorylated and dissociate from eIF4E.[45] Therefore, the inhibition of mTOR signalling by rapamycin may decrease the activity of eIF4E to initiate protein translation and result in the initiation of apoptosis. Taken together, our results suggest that mTOR and its downstream effectors, S6K1 and eIF4E play a role in the survival of CaSki cells and that the inhibition of mTOR signalling may substantially potentiate apoptosis.

In summary, the study presented here is the first to directly address the potential of targeting mTOR protein in the enhancement of therapeutic efficacy of a microtubule agent, paclitaxel, in human cervical cancer cell lines. This information may increase our understanding to apply molecular targeting therapies that may improve the management of cancer with chemotherapy.

Conflict of interest statement

None declared.

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REFERENCES

1. Ferlay J, Bray F, Pisani P, Parkin DM. *Globocan 2000: Cancer incidence, mortality, and prevalence worldwide. International*

Agency for Research on Cancer CancerBase 5, Version 1.0. Lyon: IARC; 2001.
2. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999;**83**:18–29.
3. Omura GA. Current status of chemotherapy for cancer of the cervix. *Oncology* 1992;**6**:27–32.
4. Rowinsky EK. The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. *Annu Rev Med* 1997;**48**:353–74.
5. McGuire WP, Blessing JA, Moore D, Lentz SS, Photopulos G. Paclitaxel has moderate activity in squamous cervix cancer: a Gynecologic Oncology Group study. *J Clin Oncol* 1996;**14**:792–5.
6. Wiernik PH, Schwartz EL, Strauman JJ, Dutcher JP, Lipton RB, Paietta E. Phase I clinical and pharmacokinetics study of taxol. *Cancer Res* 1987;**47**:2486–93.
7. Moore DH, Blessing JA, McQuellon RP, et al. Phase III study of cisplatin with or without paclitaxel in stage IVB, recurrent, or persistent squamous cell carcinoma of the cervix: a Gynecologic Oncology Group Study. *J Clin Oncol* 2004;**22**:3113–9.
8. Papadimitriou CA, Sarris K, Moulopoulos LA, et al. Phase II trial of paclitaxel and cisplatin in metastatic and recurrent carcinoma of the uterine cervix. *J Clin Oncol* 1999;**17**:761–6.
9. Rose PG, Blessing JA, Gershenson DM, McGehee R. Paclitaxel and cisplatin as first-line therapy in recurrent or advanced squamous cell carcinoma of the cervix: a Gynecologic Oncology Group study. *J Clin Oncol* 1999;**17**:2676–80.
10. Jordan MA, Wilson L. Microtubules and actin filaments: dynamics target for cancer chemotherapy. *Curr Opin Cell Biol* 1998;**10**:123–30.
11. Krammer PH. CD95 (APO-1/Fas)-mediated apoptosis: live and let die. *Adv Immunol* 1999;**71**:163–210.
12. Schmitz I, Kirchhoff S, Krammer PH. Regulation of death receptor-mediated apoptosis pathways. *Int J Biochem Cell Biol* 2000;**32**:1123–36.
13. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* 2002;**2**:277–88.
14. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;**281**:1309–12.
15. Li P, Nijhawan D, Budihardjo I, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997;**91**:479–89.
16. Duan H, Orth K, Chinnaiyan AM, et al. ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *J Biol Chem* 1996;**271**:16720–4.
17. Casciola-Rosen L, Nicholson DW, Chong T, et al. Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic cell death. *J Exp Med* 1996;**183**:1957–64.
18. Fraser M, Leung B, Jahani-Asl A, Yan X, Thompson WE, Tsang BK. Chemoresistance in human ovarian cancer: the role of apoptotic regulators. *Reprod Biol Endocrinol* 2003;**1**:66.
19. McCormick F. Cancer: survival pathways meet their end. *Nature* 2004;**428**:267–9.
20. Nguyen DM, Chen GA, Reddy R, et al. Potentiation of paclitaxel cytotoxicity in lung and esophageal cancer cells by pharmacologic inhibition of the phosphoinositide 3-kinase/protein kinase B (Akt). *J Thorac Cardiovasc Surg* 2004;**127**:365–75.
21. Mabuchi S, Ohmichi M, Kimura A, et al. Inhibition of phosphorylation of BAD and Raf-1 by Akt sensitizes human ovarian cancer cells to paclitaxel. *J Biol Chem* 2002;**277**:33490–500.
22. Fahy BN, Schlieman MG, Virudachalam S, Bold RJ. Inhibition of AKT abrogates chemotherapy-induced NFκB survival mechanisms: implications for therapy in pancreatic cancer. *J Am Coll Surg* 2004;**198**:591–9.
23. Kwiatkowski DJ. Rhebbing of mTOR: new insight on TSC1 and TSC2, and the pathogenesis of tuberous sclerosis. *Cancer Biol Ther* 2003;**2**:471–6.
24. Sekulic A, Hudson CC, Homme JL, et al. A direct linkage between the phosphoinositide 3-kinase-AKT signalling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res* 2000;**60**:3504–13.
25. Harris TE, Lawrence Jr JC. TOR signalling. *Science STKE.*. re 15.
26. Faried A, Faried LS, Kimura H, et al. Differential sensitivity of paclitaxel-induced apoptosis in human esophageal squamous cell carcinoma cell lines. *Cancer Chemother Pharmacol* 2006;**57**:301–8.
27. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Meth* 1991;**139**:271–9.
28. Strasser A, O'Connor L, Dixit VM. Apoptosis signalling. *Annu Rev Biochem* 2000;**69**:217–45.
29. Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* 1999;**18**:6853–66.
30. Flynn V Jr, Ramanitharan A, Moparty K, et al. Adenovirus-mediated inhibition of NF-κB confers chemosensitization and apoptosis in prostate cancer cells. *Int J Oncol* 2003;**23**:317–23.
31. Deveraux QL, Reed JC. IAP family proteins–suppressors of apoptosis. *Genes Dev* 1999;**13**:239–52.
32. Deveraux QL, Roy N, Stennicke HR, et al. IAPs block apoptosis events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* 1998;**17**:2215–23.
33. Johnson KR, Wang L, Miller 3rd MC, Willingham MC, Fan W . 5-Fluorouracil interferes with paclitaxel cytotoxicity against human solid tumor cells. *Clin Cancer Res* 1997;**3**:1739–45.
34. Lin HL, Lui WY, Liu TY, Chi CW. Reversal of Taxol resistance in hepatoma by cyclosporin A: involvement of the PI-3 kinase-AKT1 pathway. *Br J Cancer* 2003;**88**:973–80.
35. Page C, Lin HJ, Jin Y, et al. Overexpression of Akt/AKT can modulate chemotherapy-induced apoptosis. *Anticancer Res* 2000;**20**:407–16.
36. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev* 2004;**18**:1926–45.
37. Mothe-Satney I, Gautier N, Hinault C, Lawrence Jr JC, Van Obberghen E. In rat hepatocytes glucagons increases mammalian target of rapamycin phosphorylation on serine 2448 but antagonizes the phosphorylation of its downstream targets induced by insulin and amino acids. *J Biol Chem* 2004;**279**:42628–37.
38. Hinault C, Mothe-Satney I, Gautier N, Lawrence Jr JC, Van Obberghen E. Amino acids and leucine allow insulin activation of the PKB/mTOR pathway in normal adipocytes treated with wortmannin and in adipocytes from db/db mice. *FASEB J* 2004;**18**:1894–6.
39. Pham PT, Heydrick SJ, Fox HL, Kimball SR, Jefferson Jr LS, Lynch CJ. Assessment of cell-signalling pathways in the regulation of mammalian target of rapamycin (mTOR) by amino acids in rat adipocytes. *J Cell Biochem* 2000;**79**:427–41.
40. Wing LY, Chen HM, Chuang PC, Wu MH, Tsai SJ. The mammalian target of rapamycin-p70 ribosomal S kinase but not phosphatidylinositol 3-kinase-Akt signalling is responsible for fibroblast growth factor-9-induced cell proliferation. *J Biol Chem* 2005;**280**:19937–47.
41. Meric F, Hunt K. Translation initiation in cancer: a novel target for therapy. *Mol Cancer Ther* 2002;**1**:971–9.
42. Mondesire WH, Jian W, Zhang H, et al. Targeting mammalian target of rapamycin synergistically enhances chemotherapy-induced cytotoxicity in breast cancer cells. *Clin Cancer Res* 2004;**10**:7031–42.

43. Harada H, Andersen J, Mann M, Terada N, Korsmeyer S. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule Bad. *Proc Natl Acad Sci USA* 2001;**98**:9666–70.
44. Li S, Sonenberg N, Gingras AC, et al. Translational control of cell fate: availability of phosphorylation sites on translational repressor 4E-BP1 governs its proapoptotic potency. *Mol Cell Biol* 2002;**22**:2853–61.
45. Tee A, Proud C. Caspase cleavage of initiation factor 4E-binding protein I yields a dominant inhibitor of cap-dependent translation and reveals a novel regulatory motif. *Mol Cell Biol* 2002;**22**:1674–83.