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Enhancement of paclitaxel-induced microtubule stabilization, mitotic arrest, and apoptosis by the microtubule-targeting agent EM012

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Abstract

EM012, a semisynthetic phthalideisoquinoline alkaloid, has been recently found to target microtubules and possess anti-cancer activity. In this study, we evaluated the effects of EM012 in combination with the classic microtubule-targeting agent paclitaxel. Our results demonstrated that EM012 enhanced the anti-proliferative activity of nanomolar concentrations of paclitaxel in human breast cancer (MCF7), prostate cancer (DU145), and non-small-cell lung cancer (A549) cells. Further studies revealed that EM012 increased the ability of nanomolar concentrations of paclitaxel to induce mitotic arrest and apoptosis, without affecting microtubule polymerization. In contrast, when micromolar concentrations of paclitaxel were used, EM012 promoted paclitaxel-induced microtubule polymerization both in vitro and in cultured cells. Nevertheless, EM012 enhanced the ability of nanomolar concentrations of paclitaxel to stabilize microtubules, as indicated by increased tubulin acetylation. Our results therefore suggest a therapeutic potential of EM012/paclitaxel combination in the management of human cancer and provide mechanistic insights into the combined effects of these two microtubule-targeting agents.

Keywords: EM012; Paclitaxel; Microtubule; Cancer; Mitosis; Apoptosis

1. Introduction

Microtubule-targeting agents such as the vinca alkaloids (vinblastine, vincristine, vindesine, etc.) and taxanes (paclitaxel and docetaxel) are important chemotherapeutic drugs for the treatment of cancer [1,2]. The clinical use of these drugs has been hampered, however, by the side effects and limited effectiveness. The presence of diverse drug binding sites on tubulin, the subunit of microtubules, suggests that rational combinations of two or more drugs of this class might be able to enhance the anti-cancer efficacy and reduce the side effects, thereby improving the therapeutic index. This notion is supported by a number of clinical trials and preclinical studies. For example, estramustine, which binds to tubulin at a site different from the well characterized taxane site, vinca domain and colchicine site [3], has shown an ability to enhance the anti-cancer efficacy of paclitaxel and vinblastine against human prostate cancer [4–7]. Vinorelbine, a second-generation vinca alkaloid, acts synergistically with paclitaxel against leukemia and melanoma [8,9], and with docetaxel against breast cancer [10,11].

The phthalideisoquinoline alkaloid noscapine and its derivatives have been found to interfere with microtubules, and like other microtubule-targeting agents, they suppress microtubule dynamics, inhibit cell cycle progression at mitosis, and induce apoptosis [12–17]. In addition, these agents inhibit cancer progression in mouse models with little toxicity to normal tissues [12,15,18,19]. The lead compound noscapine is currently undergoing phase I/II clinical trials for cancer treatment at the University of

Abbreviations: TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; DMSO, dimethyl sulfoxide

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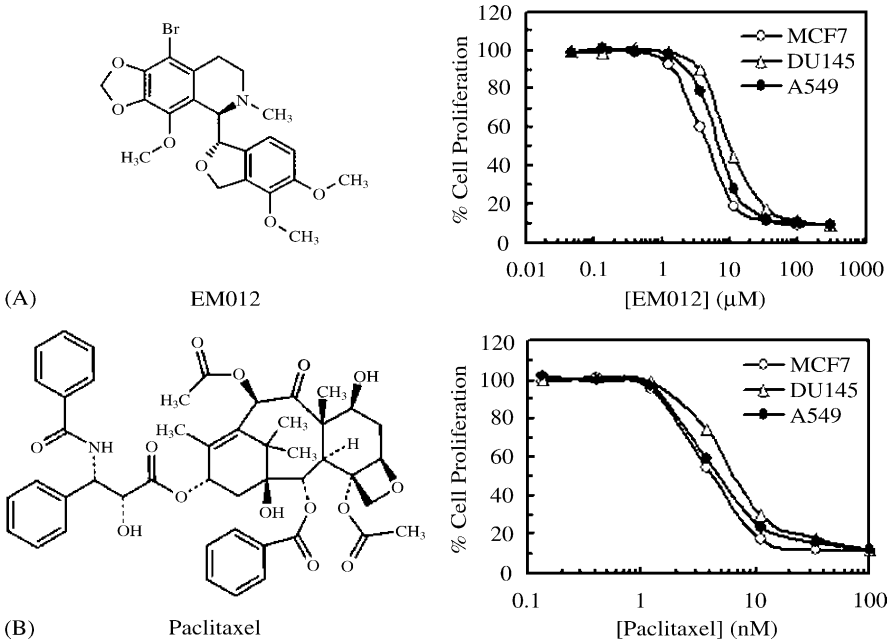

Fig. 1. Inhibition of cell proliferation by the microtubule-interfering agents EM012 (A) and paclitaxel (B). MCF7, DU145, and A549 cells were treated with individual drugs for 48 h, and the percentages of cell proliferation at indicated drug concentrations, compared to untreated cells, were measured by the in vitro cell proliferation assay as described in Section 2. Each value represents the average of three independent experiments.

Southern California, USA. EM012 (Fig. 1), a brominated derivative of noscapine, possesses 5–10-fold higher anticancer activity in comparison to noscapine in preclinical models [13,19]. In the present study, we demonstrate that EM012 significantly increases the anti-proliferative activity of paclitaxel in human breast, prostate and lung cancer cells, through enhancing microtubule stability and promoting mitotic arrest and apoptosis. The chemotherapeutic potential of EM012/paclitaxel combination in human cancer merits thorough evaluation.

2. Materials and methods

2.1. Materials

Goat brain microtubule proteins were isolated in the presence of 1 M glutamate by two cycles of polymerization and depolymerization [20]. Tubulin was purified from the microtubule proteins by phosphocellulose chromatography as described [21]. Paclitaxel was purchased from Sigma–Aldrich. EM012 (the reduced form of 5-bromonoscapine) was prepared as described previously [13,19]. Both drugs were dissolved in dimethyl sulfoxide (DMSO).

2.2. Cell culture

The human breast cancer cell line MCF7, androgen-independent prostate cancer cell line DU145, and non-small-cell ling cancer cell line A549 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen).

2.3. In vitro cell proliferation assay

Cells were seeded in 96-well plates at a density of 5×10^3 cells per well. They were treated with gradient concentrations of drugs the next day while in log-phase growth. After 48 h of drug treatment, cells were fixed with 50% trichloroacetic acid and stained with 0.4% sulforhodamine B dissolved in 1% acetic acid. Cells were then washed with 1% acetic acid to remove unbound dye. The protein-bound dye was extracted with 10 mM Tris base to determine the optical density at 564-nm wavelength. The percentage of cell survival as a function of drug concentration was then plotted to determine the IC_{50} value, which stands for the drug concentration needed to prevent cell proliferation by 50%.

2.4. Measurement of apoptosis ratio

The percentage of apoptosis was determined by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay with the in situ cell detection kit (Roche Molecular Biochemicals), following the manufacturers' instructions.

2.5. Determination of the percentage of polymeric tubulin in cells

Cells were washed with phosphate-buffered saline, and soluble proteins were then extracted under conditions that prevent microtubule depolymerization (0.1% Triton X-100, 0.1 M *N*-morpholinoethanesulfonic acid, pH 6.75, 1 mM $MgSO_4$, 2 mM EGTA, 4 M glycerol). The remain-

ing cytoskeletal fraction in the culture dish was dissolved in 0.5 ml of 0.5% SDS in 25 mM Tris (pH 6.8). Total protein concentration was then determined in each fraction by BCA reagents (Pierce). Equivalent amounts for each group were loaded for SDS/polyacrylamide gel electrophoresis. The proteins were then electrophoretically transferred for Western blotting with a mouse monoclonal anti-α-tubulin antibody (Sigma–Aldrich) and a horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson Immuno Research). Cytoskeletal (polymeric) and soluble tubulin were visualized using enhanced chemiluminescence following the manufacturer's instructions (Amersham). Their intensity was determined by densitometric analysis using a Lynx video densitometer (Biological Vision), and the percentage of polymeric tubulin in total tubulin was then calculated.

2.6. In vitro tubulin polymerization assay

Spectrophotometer cuvettes (0.4-cm path length) held a solution consisting of microtubule polymerization buffer (100 mM pipes, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP, pH 6.8) and 1 μM of EM012, 10 nM or 1 μM of paclitaxel, their combinations, or the solvent DMSO. Cuvettes were kept at room temperature before the addition of 10 μM of purified tubulin and shifted to 37 °C in a temperature controlled Ultrospec 3000 spectrophotometer (Pharmacia Biotechnology). Tubulin polymerization was monitored by measuring the changes in absorbance (350 nm) at 0.5-min intervals.

2.7. Determination of the level of tubulin acetylation

The level of tubulin acetylation was measured by Western blotting with a mouse monoclonal antibody against acetylated α-tubulin (Sigma–Aldrich) and a horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson Immuno Research). The same blot was then reprobed with the anti-α-tubulin monoclonal antibody (Sigma–Aldrich) and horseradish peroxidase-conjugated anti-mouse secondary antibody. Acetylated α-tubulin and α-tubulin were visualized, respectively, using enhanced chemiluminescence. Their intensity was determined by densitometry, and the relative level of tubulin acetylation was then calculated.

3. Results

3.1. EM012 increases the anti-proliferative activity of paclitaxel

The combined effects of the two microtubule-targeting agents, EM012 and paclitaxel, were examined in human breast cancer cell line MCF7, androgen-independent prostate cancer cell line DU145, and non-small-cell lung cancer cell line A549. The IC_{50}s for EM012 were 4.8, 10.3 and 7.1 μM in MCF7, DU145 and A549 cells, respectively (Fig. 1A). The IC_{50}s for paclitaxel were 4.2, 6.3 and 4.7 nM, respectively, in these cells (Fig. 1B). We used 0.5 and 1 μM of EM012 in the combination studies with paclitaxel; at these concentrations, EM012 by itself had nearly no effect on cell proliferation (Fig. 1A). The addition of 0.5 μM of EM012 could enhance the anti-proliferative activity of paclitaxel; however, more pronounced effects were observed when 1 μM of EM012 was used in combination (Fig. 2). For example, the addition of 1 μM of EM012 lowered the IC_{50} of paclitaxel by approximately 48% in MCF7 cells (from 4.4 to 2.3 nM), 64% in DU145 cells (from 6.1 to 2.2 nM), and 48% in A549 cells (from 4.8 to 2.5 nM) (Fig. 2).

3.2. Enhancement of paclitaxel-induced mitotic arrest by EM012

We then investigated the underlying mechanisms by which EM012 enhanced the anti-proliferative activity of paclitaxel. Because mitotic arrest is a common event in

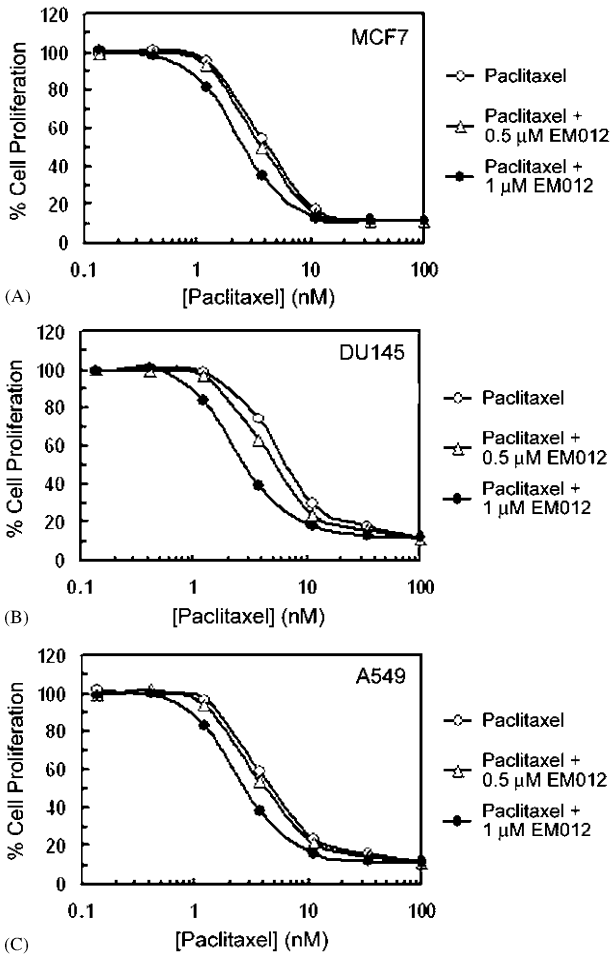

Fig. 2. EM012 enhances the anti-proliferative activity of paclitaxel in MCF7 (A), DU145 (B), and A549 (C) cells. Cells were treated for 48 h with indicated concentrations of paclitaxel in combination with 0.5 or 1 μM of EM012, and the percentages of cell proliferation were measured.

cells treated with microtubule-targeting agents including paclitaxel and EM012 [1,2], we asked whether EM012 could increase the ability of paclitaxel to induce mitotic arrest. As shown in Fig. 3, after treatment with 5 nM of paclitaxel for 24 h, MCF7, DU145 and A549 cells exhibited mitotic indices of 42, 33 and 48%, respectively. When 1 μM of EM012, which by itself had no effect on the mitotic index, was used together with 5 nM of paclitaxel, the mitotic indices were significantly increased to 66, 61 and 70% in these cells, which were close to the mitotic indices induced by 10 nM of paclitaxel (Fig. 3).

3.3. Enhancement of paclitaxel-induced apoptosis by EM012

Cells treated with microtubule-targeting agents ultimately die through the initiation of apoptosis [22]. We therefore investigated whether EM012 enhanced the antiproliferative activity of paclitaxel through increasing apoptosis induction. As shown in Fig. 4A, the addition of 1 μM of EM012 facilitated the cleavage of caspase 3 and PARP induced by paclitaxel, indicating an enhanced triggering of apoptosis. Fig. 4B shows a quantitative comparison conducted with TUNEL assay, which detects the fragmentation of DNA, a characteristic of cells undergoing apoptosis [23]. For example, MCF7, DU145 and A549 cells showed 46, 41 and 53% of apoptosis, respectively, after treatment with 5 nM of paclitaxel. The addition of 1 μM of EM012 increased paclitaxel-induced apoptosis to 76, 68 and 65% in these cells, which were close to the percentage of apoptosis induced by 10 nM of paclitaxel (Fig. 4B). In DU145 cells, the combination of 5 nM of paclitaxel and 1 μM of EM012 caused even more apoptosis than 10 nM of paclitaxel (Fig. 4B).

3.4. Combined effect of EM012 and paclitaxel on microtubule polymerization

Paclitaxel is known to promote microtubule polymerization at relatively high concentrations [24]. At lower

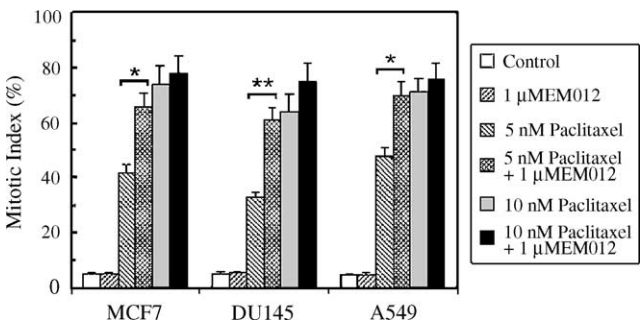

Fig. 3. Enhancement of paclitaxel-induced mitotic arrest by EM012. Cells were treated for 24 h with 1 μM of EM012, 5 or 10 nM of paclitaxel, or their combinations. The mitotic index was determined by nuclear morphology analysis. Values and error bars shown in this graph represent the averages and standard deviations, respectively, of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

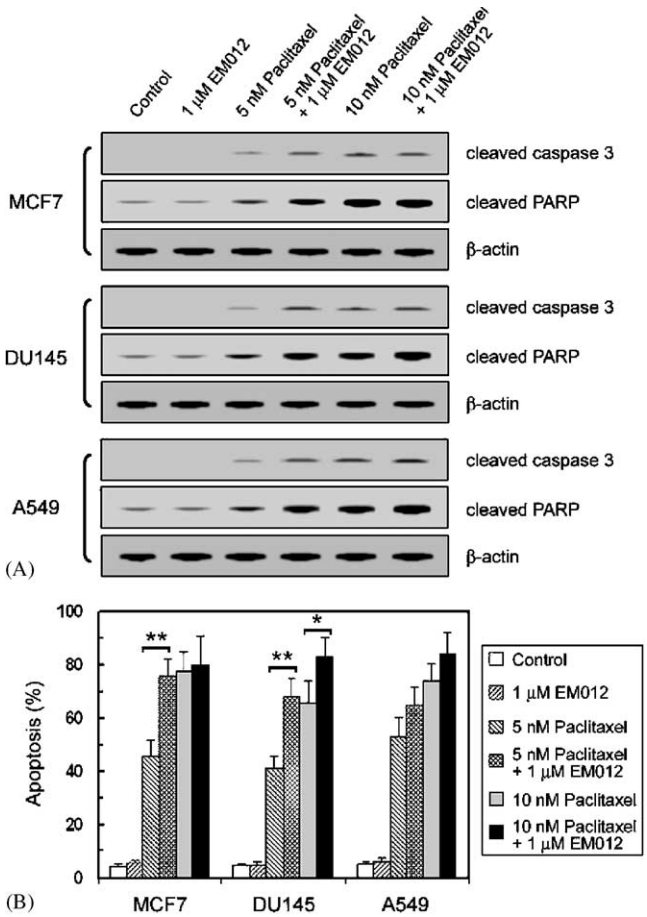

Fig. 4. Enhancement of paclitaxel-induced apoptosis by EM012. Cells were treated for 48 h with 1 μM of EM012, 5 or 10 nM of paclitaxel, or their combinations. (A) Western blot analysis of caspase 3 and PARP cleavage. β-actin was used as a loading control. (B) Percentage of apoptosis measured by TUNEL assay. Values and error bars represent the averages and standard deviations, respectively, of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

concentrations, it suppresses microtubule dynamics without affecting microtubule polymerization, but retains it ability to induce mitotic arrest and subsequent apoptosis [25–28]. Our previous drug competition studies suggested that noscapine and its derivatives such as EM012 might bind tubulin at a site different from the paclitaxel site [17]. In addition, our preliminary modeling studies suggested that noscapine and its derivatives might bind β-tubulin at the interface between the αβ-tubulin dimer, a site clearly different from the known paclitaxel site (data not shown). In this study, we examined the combined effect of EM012 and paclitaxel on microtubule polymerization and compared with the effects of individual drugs. As shown in Fig. 5A, treatment of MCF7, DU145 and A549 cells for 4 h with 1 μM of EM012, 10 nM of paclitaxel, or their combination did not have obvious effects on microtubule polymerization as measured by the percentage of polymeric tubulin. In contrast, treatment with 1 μM of paclitaxel for 4 h greatly promoted microtubule polymerization,

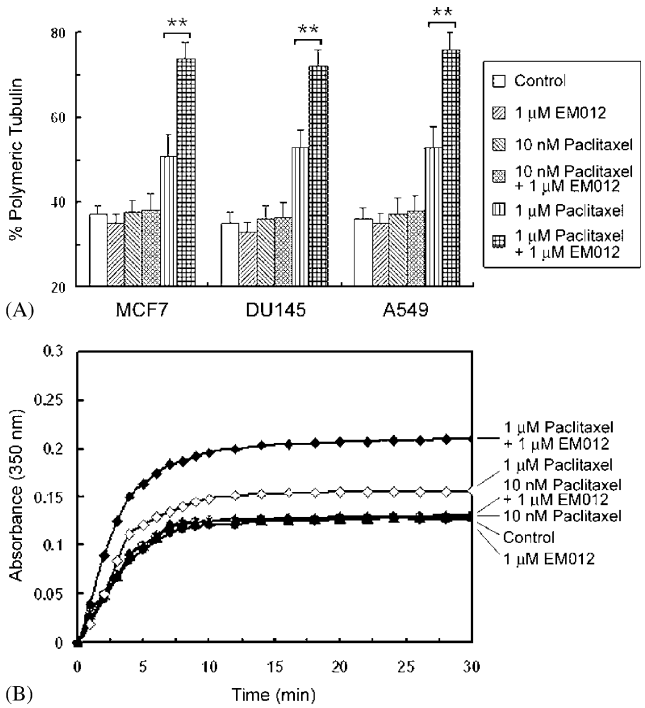

Fig. 5. Combined effect of EM012 and paclitaxel on microtubule polymerization. (A) Quantitation of the percentage of polymeric tubulin in cells treated for 4 h with 1 μM of EM012, 10 nM or 1 μM of paclitaxel, or their combinations. Values and error bars shown in this graph represent the averages and standard deviations, respectively, of three independent experiments. $^{**}P < 0.01$. (B) Effect of EM012/paclitaxel combination on the polymerization of tubulin into microtubules in vitro, measured by the changes in absorbance (350 nm).

and the effects were further enhanced by the addition of 1 μM of EM012 (Fig. 5A). The CalcuSyn program revealed that the effect of EM012 and paclitaxel on microtubule formation was synergistic (i.e., combination index lower than 1) for all the three cell lines. Consistently, immunofluorescence microscopy also showed that while 1 μM of EM012, 10 nM of paclitaxel, or their combination did not have obvious effects on microtubule morphology, 1 μM of EM012 could increase the ability of 1 μM of paclitaxel to induce microtubule bundling (data not shown).

We also investigated the effect of paclitaxel/EM012 combination on microtubule polymerization in vitro. The assay was based on the light scattering ability of polymeric tubulin, reflected as the absorbance at 350 nm of wavelength [29]. As shown in Fig. 5B, 1 μM of EM012, 10 nM of paclitaxel, or their combination had no obvious effect on the polymerization of tubulin into microtubules. However, 1 μM of paclitaxel was able to promote tubulin polymerization, similar to the effect observed in cultured MCF7, DU145 and A549 cells. Furthermore, the addition of 1 μM of EM012 enhanced the ability of 1 μM of paclitaxel to induce microtubule polymerization (Fig. 5B).

It is very intriguing that 1 μM of EM012 accelerated microtubule polymerization in the presence of 1 μM of paclitaxel, while 1 μM of EM012 itself did not induce microtubule polymerization. It is possible that 1 μM of EM012 interfered with microtubule dynamics (without affecting microtubule polymer mass), increasing the efficiency of paclitaxel binding to microtubules and thus its effect on microtubule formation.

3.5. Combined effect of EM012 and paclitaxel on microtubule stability

In Fig. 2, we showed that 1 μM of EM012 enhanced the ability of nanomolar concentrations of paclitaxel to inhibit cell proliferation. Interestingly, although 1 μM of EM012 accelerated microtubule polymerization in the presence of 1 μM of paclitaxel, it did not promote microtubule polymerization in the presence of 10 nM of paclitaxel (Fig. 5). These results suggested that the enhanced anti-proliferative activity of 1 μM of EM012 with nanomolar concentrations of paclitaxel was probably independent of microtubule polymerization. It is possible that, as discussed above, 1 μM of EM012 suppressed microtubule dynamics (with-

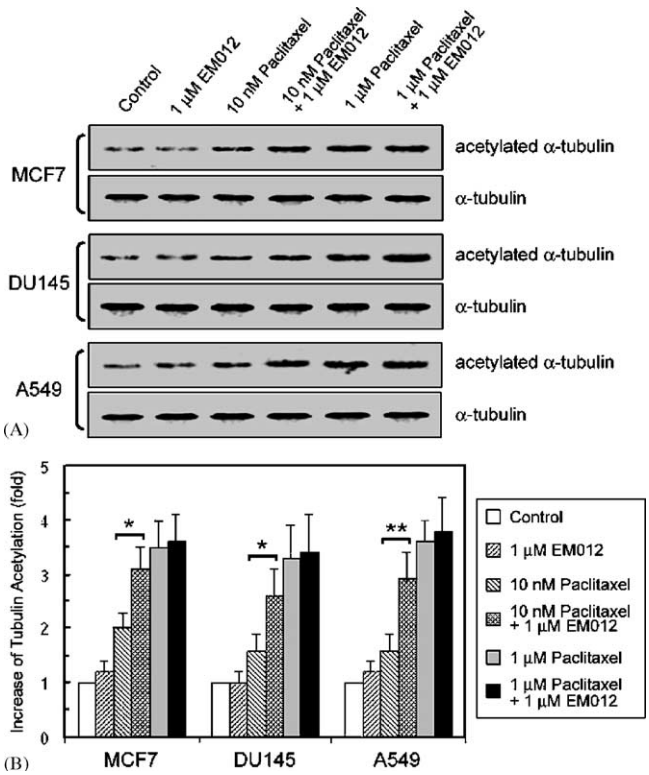

Fig. 6. Combined effect of EM012 and paclitaxel on tubulin acetylation as a measure of microtubule stability. (A) Western blot analysis showing the level of tubulin acetylation in cells treated for 4 h with 1 μM of EM012, 10 nM or 1 μM of paclitaxel, or their combinations. (B) Fold increase of tubulin acetylation. Cells were treated as described above and the level of tubulin acetylation was measured and quantified by densitometry as described in Section 2. Values and error bars represent the averages and standard deviations, respectively, of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

out affecting microtubule polymer mass), allowing for more efficient paclitaxel binding to microtubules. This might in turn increase the ability of 10 nM of paclitaxel to 'stabilize' microtubules. However, for EM012 to increase paclitaxel-induced microtubule 'formation', 10 nM of paclitaxel might not be sufficient, while 1 μM of paclitaxel might have passed the threshold.

We tested whether 1 μM of EM012 enhanced the ability of 10 nM of paclitaxel to stabilize microtubules by examining the level of α-tubulin acetylation, a marker of microtubule stability [30–32]. The level of acetylated α-tubulin is found to be correlated with the stability of microtubules, and paclitaxel is also well known to increase tubulin acetylation [30–32]. However, it remains mysterious whether tubulin acetylation is a cause or consequence of microtubule stability as well as how paclitaxel induces tubulin acetylation.

We found that addition of 1 μM of EM012 significantly increased tubulin acetylation induced by 10 nM of paclitaxel in all three cell lines (Fig. 6). For example, in A549 cells, 10 nM of paclitaxel increased tubulin acetylation by 1.6-fold, and the combination of 10 nM of paclitaxel and 1 μM of EM012 increased tubulin acetylation by 2.9-fold. Interestingly, however, 1 μM of EM012 had only subtle effect on the level of tubulin acetylation induced by 1 μM of paclitaxel (Fig. 6). At first glance this result seemed to be contradictory with the synergistic effects of EM012 and paclitaxel in inducing microtubule polymerization (Fig. 5), which indicated that they might have a pronounced effect on tubulin acetylation. This result is not unreasonable, however, since the level of tubulin acetylation induced by 1 μM of paclitaxel might have almost reached the plateau and could not be significantly enhanced further by EM012.

Taken together, these results suggested that EM012 might enhance the anti-proliferative activity of nanomolar concentrations of paclitaxel through increasing microtubule stability.

4. Discussion

The microtubule cytoskeleton represents one of the best targets to date for cancer chemotherapy. The effectiveness of microtubule-targeting drugs has been validated by the successful use of the vinca alkaloids and taxanes for the treatment of a wide variety of human cancers. For example, paclitaxel, a complex diterpene derived from the Pacific yew *Taxus brevifolia*, is used in the clinic against breast cancer, ovarian cancer, non-small-cell lung cancer, Kaposi's sarcoma, and several other malignancies. Unfortunately, the clinical applicability of paclitaxel, as well as other microtubule-targeting chemotherapeutics, has been impaired by the side effects, notably neurological and hematological toxicities, which are probably due to the inhibition of axonal transport and the inhibition of the rapidly dividing hematopoietic cells [33]. On the other hand, strategies exploiting rational drug combinations have shown a great potential in enhancing the anti-cancer efficacy and lowering the side effects of the individual drugs [2].

As our knowledge of microtubule-targeting drugs increases, we recognize that the mechanism underlying the anti-cancer activity of these agents may primarily lie in their inhibitory effects on microtubule dynamics, rather than in their effects on microtubule polymer mass. Chemical compounds that weakly bind to tubulin and suppress microtubule dynamics without significantly affecting microtubule polymer mass, such as estramustine [3], noscapine [14], and dicoumarol [34], are expected to display anti-cancer activity without causing deleterious toxicity to normal tissues. Microtubule-targeting drugs that suppress microtubule dynamics by different mechanisms may be used in combination for cancer chemotherapy. Especially, the combination of conventional microtubule-targeting drugs with weak tubulin-binding agents as discussed above, may improve the anti-cancer efficacy and minimize the toxicities.

EM012, a phthalideisoquinoline alkaloid derived from noscapine, weakly binds to tubulin with a dissociation constant of 106 μM, and possesses more potent activity than noscapine in inhibiting cancer cell proliferation [13,19]. In a mouse model of human ovarian cancer, EM012 does not cause obvious side effects while inhibiting cancer progression [19]. In this study, we demonstrate that with a concomitant exposure of 48 h, EM012 enhances the anti-proliferative activity of paclitaxel at lower dosages in human breast, prostate and non-small-cell lung cancer cells. In addition, our preliminary studies indicate that EM012-mediated sensitization does not dependent on the schedule of combination (data not shown). Our data also suggest that the enhanced anti-proliferative activity of the EM012/paclitaxel combination may result from the synergistic/additive suppression of microtubule dynamics, as reflected by the increased microtubule stability. The synergistic/additive suppression of microtubule dynamics may in turn cause the increased mitotic arrest and apoptosis observed in these cancer cells.

Taken together, our results presented in the current study provide a rationale for the combination of EM012 and paclitaxel for cancer treatment. It will be of great interest to investigate in the future the effect of the EM012/paclitaxel combination on cancer progression in animal models. Clinical trials will certainly be required to provide a definite answer to the applicability of the EM012/paclitaxel combination for cancer therapy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2004.08.032.

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