

# Identification of a lathyrane-type diterpenoid EM-E-11-4 as a novel paclitaxel resistance reversing agent with multiple mechanisms of action

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## ABSTRACT

P-glycoprotein (P-gp) and  $\beta$ III-tubulin overexpression-mediated drug resistance leads to clinical therapy failure for paclitaxel. However, the development of paclitaxel-resistance reversal agents has not had much success. In this study, EM-E-11-4, a lathyrane-type diterpenoid extracted from *Euphorbia micractina*, demonstrated good anti-MDR (multidrug resistance) activity in paclitaxel-resistant tumor cells overexpressing either P-gp or  $\beta$ III-tubulin. EM-E-11-4 was able to recover the effects of paclitaxel in inducing arrest at G<sub>2</sub>/M phase and apoptosis in both A549/Tax (P-gp overexpression) and Hela/ $\beta$ III ( $\beta$ III-tubulin overexpression) cells, respectively, at a non-cytotoxic dose. EM-E-11-4 could enable Flutax-1 and Rhodamine 123 be accumulated intracellularly at an accelerating rate in A549/Tax cells by inhibiting the activity of P-gp ATPase, rather than affecting the expression of P-gp. In addition, it also strengthened the effects of paclitaxel in promoting tubulin polymerization and the binding of paclitaxel to microtubules *in vitro*. It inhibited the expression of  $\beta$ III-tubulin in Hela/ $\beta$ III cells in a dose-dependent manner while not exerting influence on the other  $\beta$ -tubulin subtypes. As far as we know, this is the first study to report that a small molecule natural product could specifically inhibit the expression of  $\beta$ III-tubulin. These results suggest EM-E-11-4 may serve as a promising MDR reversal agent, particularly for patients bearing tumors with high expression of P-gp and  $\beta$ III-tubulin.

## INTRODUCTION

Chemotherapy is an alternative therapeutic method for patients with cancer. Nevertheless, the outcome is not always satisfactory because drug resistance can develop during the course of treatment. As a first-line chemotherapy, paclitaxel has been used to treat patients suffering from breast and ovarian cancer for decades [1–3]. However, the intrinsic and acquired tumor drug resistance severely restrained its clinical application [4–5], which was observed in cell lines correlated to overexpression of P-glycoprotein (P-gp) and  $\beta$ III-tubulin [6–8].

As a vital transporter of the ATP-binding cassette (ABC) family [9–10], P-gp is encoded by *abcb1/mdr1* gene [11], functioning as an ATP-dependent broad-spectrum drug efflux pump, and the drug concentration in cells is down-regulated by P-gp. It is involved with lots of structurally uncorrelated anti-cancer drugs, for instance, paclitaxel, docetaxel, doxorubicin, and vincristine [12], and that can lead to multidrug resistance (MDR). The relationship between cancer chemotherapy resistance and P-gp expression has been confirmed in numerous clinical studies [13–15]. In recent years, there has been a certain degree of success in the development of P-gp-mediated paclitaxel resistance reversal agents [16–18].

Many clinical studies have provided evidence that  $\beta$ III-tubulin overexpression results in another type of paclitaxel resistance in tumor cells [19–21]. Generally, mainly in neuronal cells,  $\beta$ III-tubulin is expressed and it is rarely detectable in other tissues. However,  $\beta$ III-tubulin has abnormally high expression in certain drug-resistant tumor cells originating from breast, lung, prostate, and stomach tissues [19, 22]. The exact mechanism of this type of resistance is not fully elucidated.

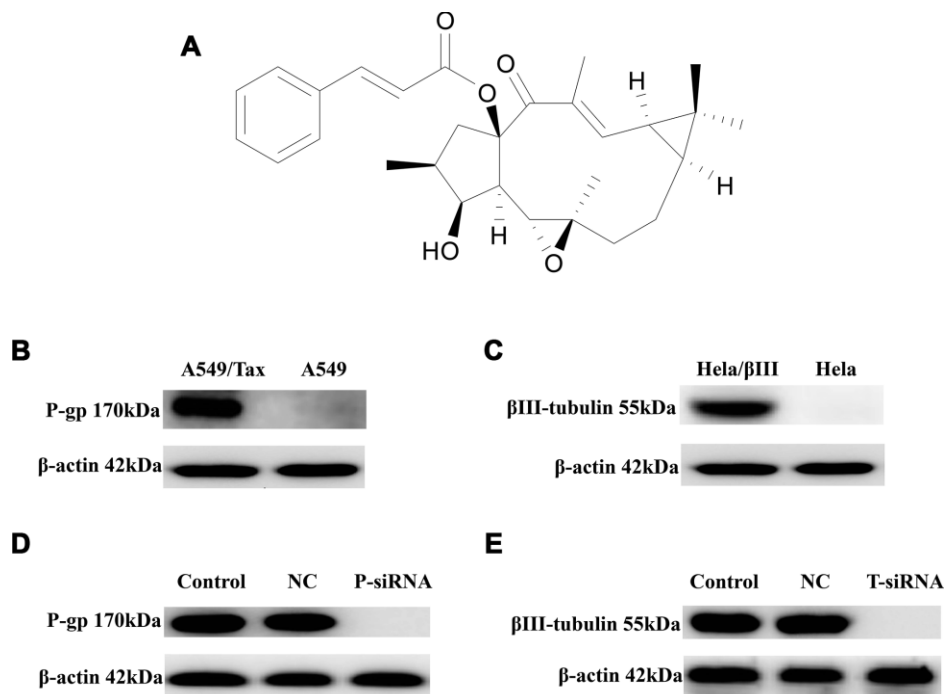
To overcome the drug resistance mentioned above, our lab pursued novel MDR reversal agents from natural products that can resume the sensitivity to chemotherapy drugs for MDR tumor cells. Among these compounds, EM-E-11-4 is a lathyrane-type diterpenoid from *Euphorbia micractina* that could markedly reverse the sensitivity of drug-resistant cells from different tissues to paclitaxel at its concentration without cytotoxicity. Those tissues we investigated include the human lung adenocarcinoma cell line A549 and its P-gp overexpression drug-resistant counterpart A549/Tax, the same as the paclitaxel-resistant cell line HeLa/ $\beta$ III and the human cervical cell line HeLa. HeLa/ $\beta$ III is originated from  $\beta$ III-tubulin gene transfection. Therefore, we also explored the mechanism of action for the effectiveness of EM-E-11-4 in MDR reversal.

## RESULTS

### Reversal effect of EM-E-11-4 in drug-resistance cells

The cytotoxicity of EM-E-11-4 was examined by an MTT assay in A549/Tax (overexpression of P-gp, Figure 1B) and HeLa/ $\beta$ III (overexpression of  $\beta$ III-tubulin, Figure 1C) cell lines. As shown in Table 1, EM-E-11-4 at 20–30  $\mu$ M exerted considerable cytotoxicity. In the MDR cell lines, A549/Tax and HeLa/ $\beta$ III demonstrated the same sensitivity to EM-E-11-4 as their parental cells. More than 90% of cells survived at a concentration of 10  $\mu$ M EM-E-11-4 in all assays. According to the results from the cytotoxicity assay mentioned above, EM-E-11-4 at 2.5, 5, and 10  $\mu$ M was chosen to evaluate the reversal activity. As shown in Table 2, EM-E-11-4 markedly decreased IC<sub>50</sub> values for paclitaxel in A549/Tax, HeLa/ $\beta$ III, and their parental cells. EM-E-11-4 strengthened the effect of paclitaxel better than verapamil in A549/Tax cells, and it had similar effects in HeLa/ $\beta$ III cells. These results indicate that EM-E-11-4 could reverse paclitaxel-resistance mediated through P-gp or  $\beta$ III-tubulin.

Through cell transfection with siRNAs targeting P-gp or  $\beta$ III-tubulin, the expression level of P-gp or  $\beta$ III-tubulin was suppressed (Figure 1D and 1E). With or without



**Figure 1. The expression of P-gp and  $\beta$ III-tubulin in cells.** (A) Chemical structure of EM-E-11-4. (B) P-gp levels in A549 and A549/Tax cells. (C)  $\beta$ III-tubulin levels in HeLa and HeLa/ $\beta$ III cells. (D) The effect of P-gp siRNA on P-gp expression in A549/Tax cells. (E) The effect of TUBB3 siRNA on  $\beta$ III-tubulin expression in HeLa/ $\beta$ III cells. Cells were treated with vehicle (negative control, NC) or siRNAs (P-gp siRNA, TUBB3-siRNA), and protein levels were determined by Western blot analysis.

**Table 1. Cytotoxic activities of EM-E-11-4 against various human tumor cell lines.**

Compound	IC <sub>50</sub> (μM, Mean ± SD)		IC <sub>50</sub> (μM, Mean ± SD)	
	A549	A549/Tax	Hela	Hela/βIII
EM-E-11-4	31.5 ± 2.3	40.2 ± 2.0	21.1 ± 4.6	25.3 ± 5.8

Data are presented as mean ± SD from three independent experiments.

**Table 2. Cytotoxic activity of paclitaxel combined with EM-E-11-4 against various human tumor cell lines.**

Cell lines	IC <sub>50</sub> (nM, Mean ± SD)/ Reverse Index				
	Paclitaxel	+EM-E-11-4 (2.5μM)	+EM-E-11-4 (5μM)	+EM-E-11-4 (10μM)	+Vrp (10μM)
A549	4.7±1.0	3.3±0.6 (1.4)	0.59±0.08 (8.0)	0.41±0.12 (11.5)	4.3±0.9 (/)
A549/Tax	1559±86	157±13.4 (9.9)	56.7±7.7 (27.5)	22.9 ± 4.7 (68.1)	64.7±7.6 (24.1)
Hela	4.3±0.4	2.8 ± 0.42 (1.5)	1.4±0.3 (3.1)	0.63±0.11 (6.8)	/
Hela/β-III	52.9±4.9	4.6±1.0 (11.5)	3.6±0.8 (14.7)	1.8±0.4 (29.4)	/

Reverse Index = IC<sub>50</sub> (paclitaxel)/IC<sub>50</sub> (paclitaxel+EM-E-11-4)

Vrp, verapamil. Data are presented as mean ± SD from three independent experiments.

**Table 3. Cytotoxic activity of paclitaxel combined with EM-E-11-4 against A549/Tax (P-gp siRNA) and Hela/βIII (TUBB3 siRNA) cell lines.**

Compound	IC <sub>50</sub> (nM, Mean ± SD)		IC <sub>50</sub> (nM, Mean ± SD)	
	A549/Tax		Hela/βIII	
	NC	P-siRNA	NC	T-siRNA
Paclitaxel	1498±35	24.4±3.6	48.7±4.3	2.4±0.3
Paclitaxel + EM-E-11-4 10μM	25.4±3.2	22.5±2.7	2.1±0.4	2.3±0.4

Data are presented as mean ± SD from three independent experiments.

EM-E-11-4 treatment, the activity of paclitaxel in the siRNA transfected cells was respectively assessed. As Table 3 shows, suppressed expression of P-gp or βIII-tubulin through siRNA increases the sensitivity of A549/Tax and Hela/βIII cells to paclitaxel. However, EM-E-11-4 did not dramatically influence the IC<sub>50</sub> of paclitaxel in those cells. All the results confirm that EM-E-11-4 could reverse drug resistance by suppressing the functions of P-gp or βIII-tubulin.

#### **EM-E-11-4 strengthened the effect of paclitaxel-induced G<sub>2</sub>/M phase arrest and apoptosis in A549/Tax and Hela/βIII cells**

It is well known that paclitaxel can trigger cell arrest in G<sub>2</sub>/M phase and apoptosis by disturbing microtubule function, whereas the overexpression of βIII-tubulin and P-gp suppressed these effects. A549/Tax and Hela/βIII cells were treated with paclitaxel (A549/Tax: paclitaxel 100 nM; Hela/βIII: paclitaxel 20 nM) alone or in combination with EM-E-11-4 for 24 h (Supplementary Figure 1 and Supplementary Figure 2). EM-E-11-4 did not affect the cell cycle of A549/Tax and Hela/βIII cells alone. However,

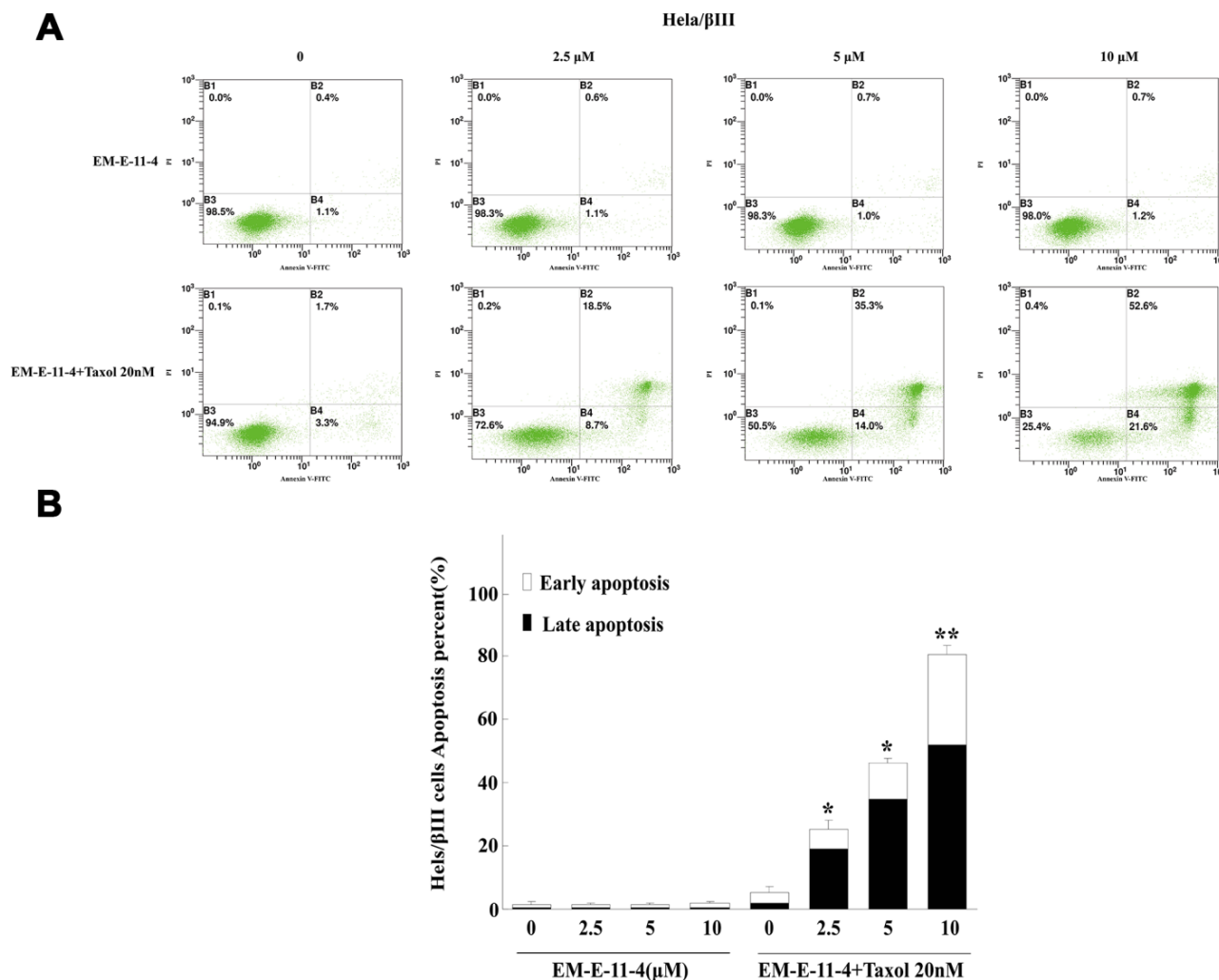
paclitaxel combined with EM-E-11-4 (2.5, 5, and 10 μM) increased the percentage of G<sub>2</sub>/M phase from 31.7% (100 nM paclitaxel alone) to 42.7%, 45.7%, and 60.5% in A549/Tax cells (Supplementary Figure 1). It increased from 40.1% (20 nM paclitaxel alone) to 52.7%, 53.7%, and 58.1% in Hela/βIII cells (Supplementary Figure 2). At the same time, EM-E-11-4 increased the percentage of paclitaxel-induced apoptosis for 48 h. Paclitaxel combined with EM-E-11-4 (2.5, 5, and 10 μM) increased the percentage of apoptotic cells from 21.9% (100 nM paclitaxel alone) to 34.6%, 40.2%, and 44.2% in A549/Tax cells (Figure 2). It increased from 5.23% (20 nM paclitaxel alone) to 27.76%, 49.08%, and 76.92% in Hela/βIII cells (Figure 3). These results indicate that EM-E-11-4 could enhance the effect of paclitaxel-induced G<sub>2</sub>/M phase arrest and apoptosis in cells overexpressing P-gp and βIII-tubulin in a dose-dependent manner.

#### **EM-E-11-4 accelerated the accumulation of Flutax-1 and Rhodamine 123 in A549/Tax cells**

P-gp is characterized by a decrease in accumulation and enhancement of the efflux of anticancer drugs, whose

effects can be reversed through P-gp inhibitors, for example, verapamil. To investigate the mechanism in which EM-E-11-4 reverses P-gp-mediated paclitaxel resistance in tumors, a P-gp overexpressing cell line, A549/Tax, was used as the drug-resistant model. Flutax-1 is a taxane coupled with a fluorescent group that can bind to the taxol-binding site of  $\beta$ -tubulin and be used as a P-gp substrate. After incubation with 5  $\mu$ M Flutax-1 alone or in combination with EM-E-11-4 for 3 h, A549 and A549/Tax cells were observed by fluorescence microscope (Figure 4A). A549 cells exhibited a normal network of microtubules (MTs) with clear green fluorescence, which indicates that Flutax-1 entered the cell and bound to the taxol-binding site of MTs (Figure 4A-a). A549/Tax cells exhibited weak fluorescence, which indicates that P-gp mediates the efflux of Flutax-1 (Figure 4A-b). EM-E-11-4 dose-

dependently increased Flutax-1 accumulation in A549/Tax cells (Figure 4A-c, d and e). After treated A549/Tax cells with Flutax-1 and 10  $\mu$ M EM-E-11-4, they exhibited network of MTs that seemed clearer than the parallel assay using 10  $\mu$ M verapamil (Figure 4A-f). After treatment with Rhodamine 123 (Rh123) and EM-E-11-4 for 0.5 h in A549 and A549/Tax cells, the level of intracellular Rh123 was monitored using flow cytometry (Figure 4B). In A549 cells, EM-E-11-4 made no difference on the concentration of intracellular Rh123 (Figure 4B-a and c). However, in A549/Tax cells, EM-E-11-4 dose-dependently increased the level of intracellular Rh123 (Figure 4B-b and d). These results suggest EM-E-11-4 could reverse P-gp-mediated drug resistance through suppressing the efflux function of P-gp, and it might accelerate the accumulation of paclitaxel in A549/Tax cells.

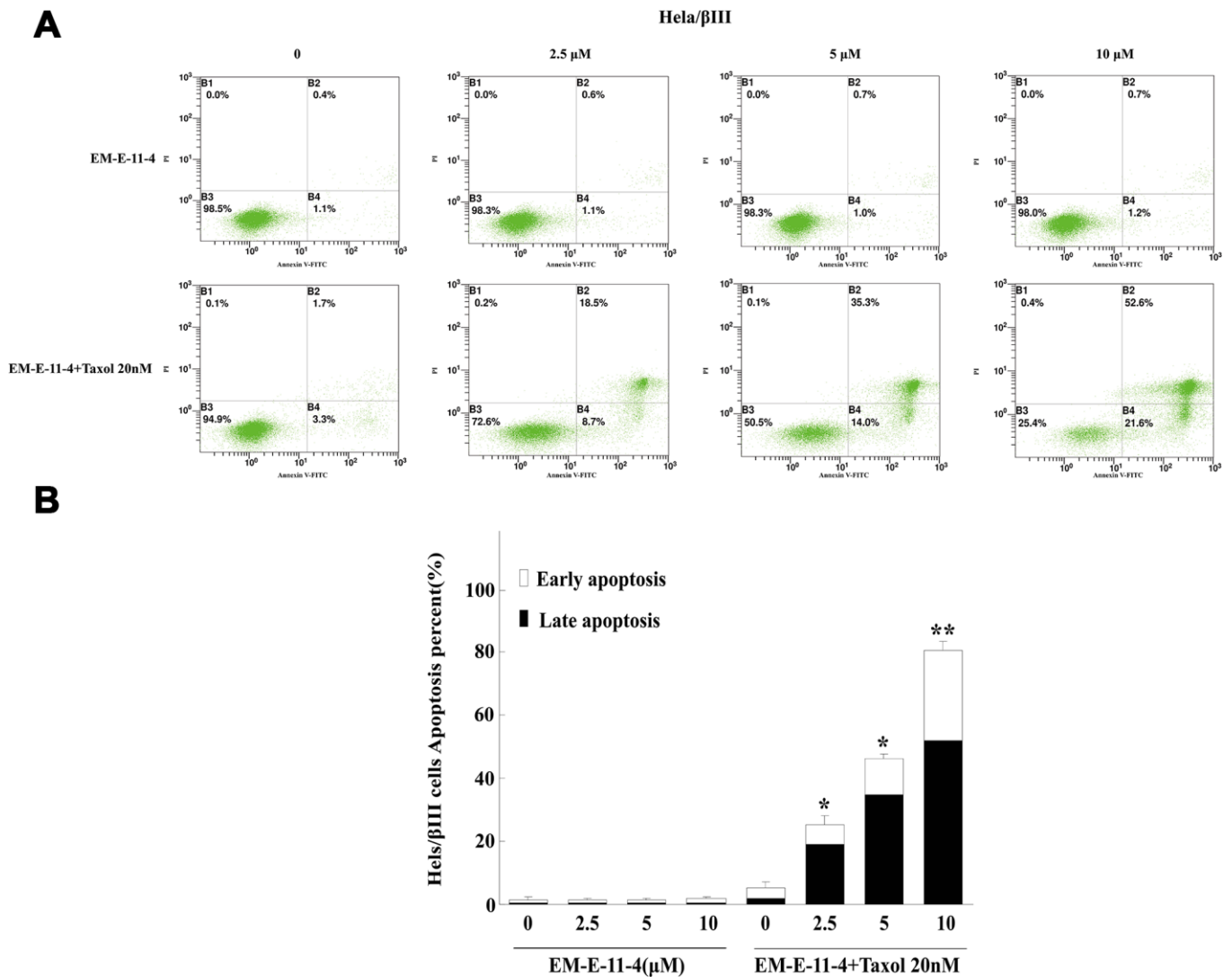


**Figure 2. Apoptosis as detected by Annexin V-FITC/PI binding assay (48 h) in A549/Tax cells.** (A) Apoptosis induction by EM-E-11-4 (2.5, 5, and 10  $\mu$ M) combined with or without 100 nM paclitaxel in A549/Tax cells. (B) The percent apoptosis in A549/Tax cells. Columns represent the means $\pm$ SD values for apoptotic cells obtained from three individual experiments. \*  $p < 0.05$  and \*\*  $p < 0.01$  vs. control (10  $\mu$ M EM-E-11-4).

**EM-E-11-4 inhibits the ATPase activity of P-gp without affecting the expression of P-gp**

After treated A549/Tax cells with EM-E-11-4 for 48 h, the expression level of P-gp had no marked change (Figure 5A). However, EM-E-11-4 did affect the ATPase activity of P-gp in the Pgp-Glo™ Assay System. As a substrate of P-gp, paclitaxel binds to P-gp and is transported through the protein. Thus, with the increasing consumption of ATP, which was assessed as a change in luminescence, the ATPase activity was demonstrated to be increase. Na<sub>3</sub>VO<sub>4</sub> inhibits the P-gp ATPase activity, and with the existence of Na<sub>3</sub>VO<sub>4</sub>, the data were normalized refer to the ATP consumption. As Figure 5B shows, small change in luminescence was detected with the untreated vehicle control and EM-E-11-4 (2, 10, and

50 μM), which indicates low ATP consumption. This reflects the weak intrinsic basal ATPase activity of P-gp. Adding paclitaxel (50 μM) led to increasingly higher ATP consumption, which was reflected in the stimulation of P-gp ATPase activity. With the existence of paclitaxel combined with EM-E-11-4 (2, 10, and 50 μM), ATP consumption was actually lower than in the presence of paclitaxel alone, which indicates a lower level of ATPase consumption. With the existence of paclitaxel combined with 50 μM EM-E-11-4, ATP consumption was lower than when it was combined with 100 μM verapamil. These results suggest that EM-E-11-4 hinders P-gp ATPase activity that results in a decrease in the efflux of intracellular paclitaxel by P-gp. The results partly illustrate its circumvention function of P-gp-mediated drug resistance.



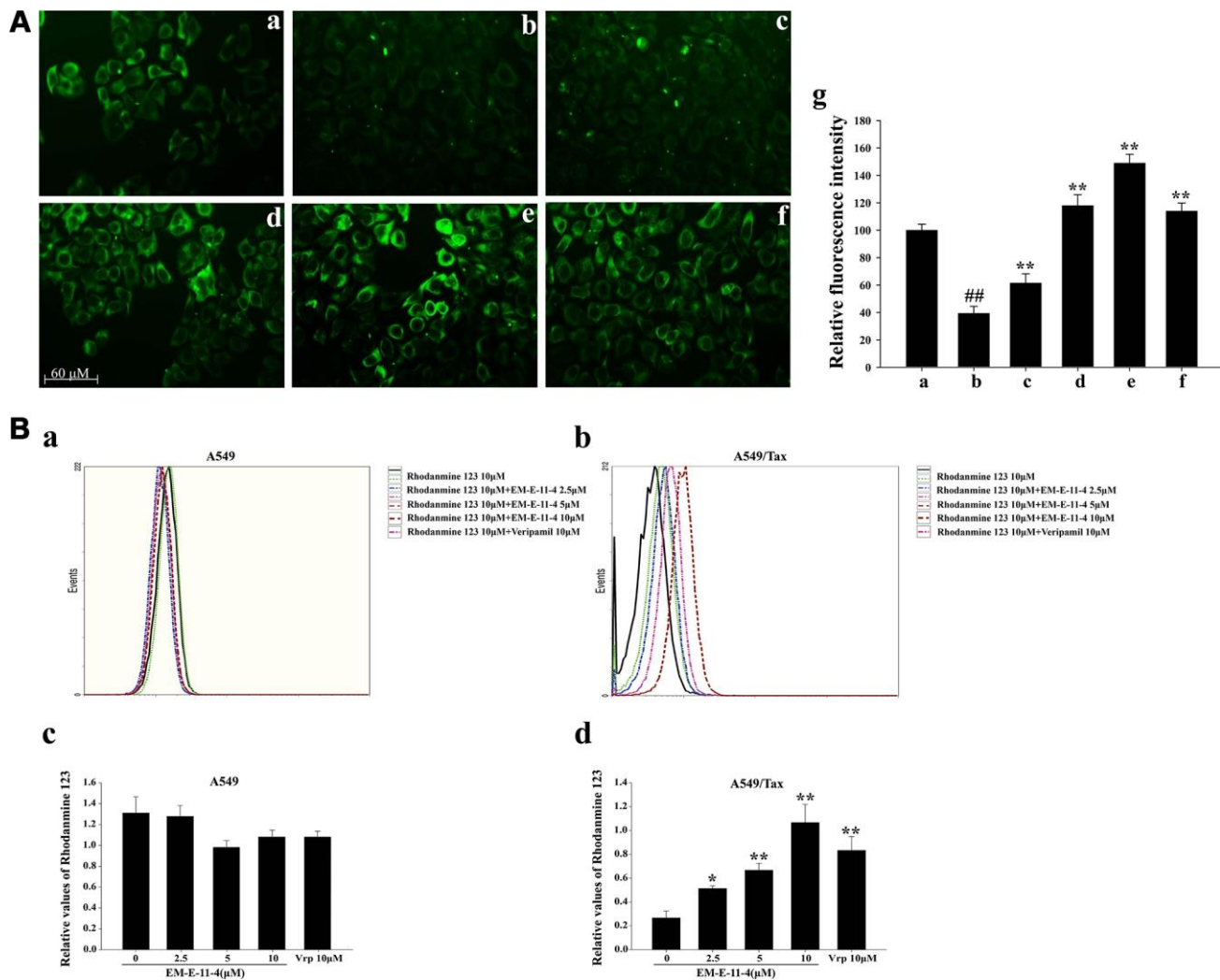
**Figure 3. Apoptosis detected by Annexin V-FITC/PI binding assay (48 h) in HeLa/βIII cells. (A)** Apoptosis induction by EM-E-11-4 (2.5, 5, and 10 μM) combined with or without 20 nM paclitaxel in HeLa/βIII cells. **(B)** The percent apoptosis of HeLa/βIII cells. Columns represent the means±SD values for apoptotic cells obtained from three individual experiments. \* *p*<0.05 and \*\* *p*<0.01 vs. control (10μM EM-E-11-4).



## EM-E-11-4 strengthened the effect of paclitaxel-induced tubulin polymerization *in vitro*

As shown in Figure 6, paclitaxel could promote tubulin polymerization *in vitro* since the tubulin polymerization levels are reflected by the fluorescence intensity. EM-E-11-4 dose-dependently strengthened the effect of paclitaxel-induced tubulin polymerization, whereas EM-E-11-4 alone did not induce tubulin polymerization (Figure 6A). Western blot analysis indicated that EM-E-11-4 has no influence on the soluble and insoluble states of MTs in

A549/Tax and HeLa/ $\beta$ III cells. However, EM-E-11-4 combined with paclitaxel markedly increased the proportion of MTs in the insoluble states more than paclitaxel alone (Figure 6B and 6C). Our study also shows that EM-E-11-4 promotes binding constant of paclitaxel for MTs (Supplementary Table 1). These results indicate EM-E-11-4 was capable of enhancing the effect of paclitaxel-induced tubulin polymerization and the binding of paclitaxel to MTs. It is one of the reasons why it strengthened the effect of paclitaxel-induced G<sub>2</sub>/M phase arrest and apoptosis, causing reverse drug resistance in cancer cells.

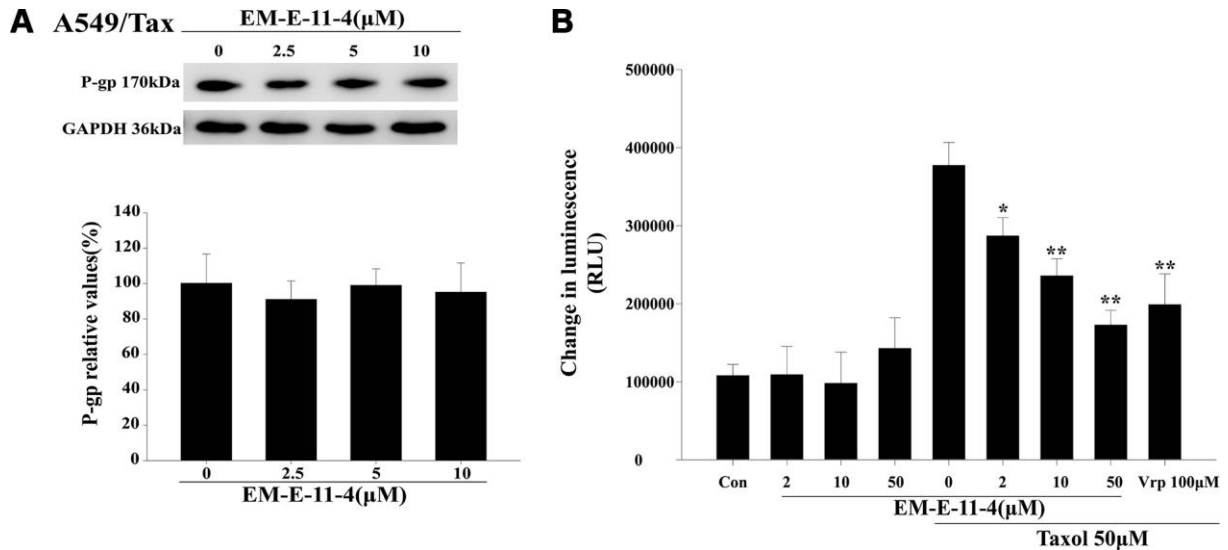


**Figure 4. Effects of EM-E-11-4 on the accumulation of Flutax-1 and Rhodamine 123 in A549 and A549/Tax cells. (A)** Effects of EM-E-11-4 on the accumulation of Flutax-1 in A549 and A546/Tax ( $\times 100$ ) cells. **(A-a)** 5  $\mu$ M Flutax-1 in A549 cells; **(A-b)** 5  $\mu$ M Flutax-1 in A549/Tax cells; **(A-c)** 5  $\mu$ M Flutax-1 and 2.5  $\mu$ M EM-E-11-4 in A549/Tax cells; **(A-d)** 5  $\mu$ M Flutax-1 and 5  $\mu$ M EM-E-11-4 in A549/Tax cells; **(A-e)** 5  $\mu$ M Flutax-1 and 10  $\mu$ M EM-E-11-4 in A549/Tax cells; **(A-f)** 5  $\mu$ M Flutax-1 and 10  $\mu$ M verapamil in A549/Tax cells; **(A-g)** quantitative data for the fluorescence intensity. ##  $p < 0.01$  vs. A549 cells control, \*\*  $p < 0.01$  vs. A546/Tax cells control. **(B)** Effects of EM-E-11-4 on the accumulation of Rhodamine 123 in A549 and A549/Tax cells. **(B-a)** The levels of Rhodamine 123 assayed by flow cytometry in A549 cells; **(B-b)** The levels of Rhodamine 123 assayed by flow cytometry in A549/Tax cells; **(B-c)** The relative values of Rhodamine 123 in A549 cells; **(B-d)** The relative values of Rhodamine 123 in A549/Tax cells. Columns represent the means  $\pm$  SD values for Rhodamine 123 obtained from three individual experiments. \*  $p < 0.05$  and \*\*  $p < 0.01$  vs. control (10  $\mu$ M EM-E-11-4).

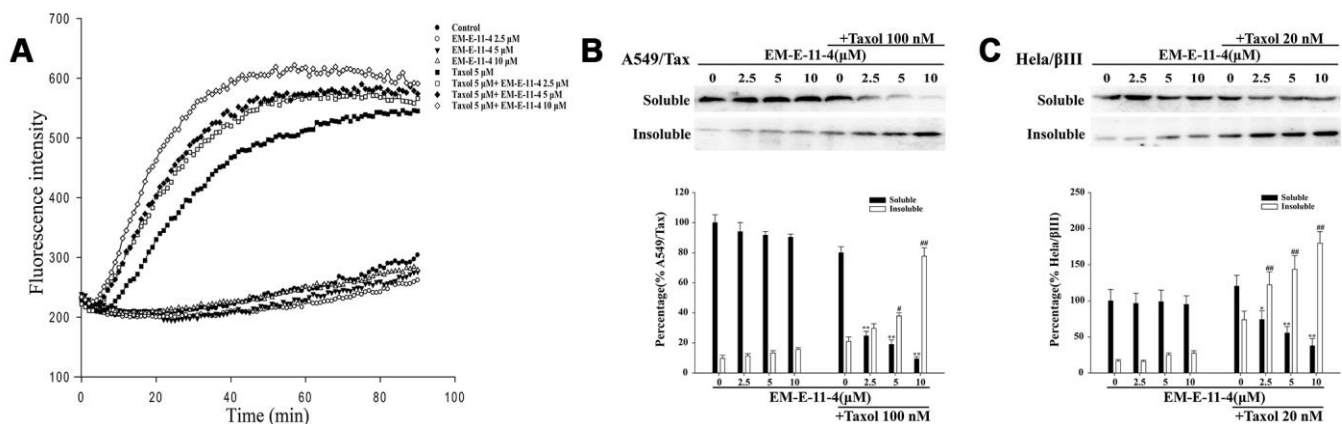
## EM-E-11-4 inhibited the expression of $\beta$ III-tubulin in HeLa/ $\beta$ III cells

We found EM-E-11-4 decreases the level of  $\beta$ III-tubulin expression in HeLa/ $\beta$ III cells (Figure 7). After treating HeLa/ $\beta$ III cells with EM-E-11-4 for 48 h, Western blot

(Figure 7A) and immunofluorescence analysis (Figure 7B) indicated that the expression of  $\beta$ III-tubulin was decreased by EM-E-11-4 in a dose-dependent manner. This suggests that inhibition of  $\beta$ III-tubulin expression was the most important function of EM-E-11-4 to overcome  $\beta$ III-tubulin-mediated paclitaxel resistance in tumors.



**Figure 5. EM-E-11-4 inhibited the P-gp ATPase activity.** (A) A549/Tax cells were treated with EM-E-11-4 (2.5, 5, and 10  $\mu$ M) for 48 h, and P-gp was determined by Western blot analysis. (B) A luminescent assay for P-gp ATPase activity was performed according to the Pgp-Glo™ Assay System instructions. ATP consumption in the presence EM-E-11-4 (2, 10, and 50  $\mu$ M) combined with or without paclitaxel. Con: control; Vrp: verapamil. Columns represent the means $\pm$ SD values for the luminescent change obtained from three individual experiments \*  $p < 0.05$  and \*\*  $p < 0.01$  vs. control (10  $\mu$ M EM-E-11-4).



**Figure 6. EM-E-11-4 enhanced the effect of paclitaxel-induced tubulin polymerization *in vitro*.** (A) Tubulin polymerization assay. Purified porcine tubulin in reaction buffer was incubated at 37°C with GTP in the absence or presence of the indicated agents. Tubulin polymerization was measured using a fluorescence microplate reader (ex = 370 nm, em = 445 nm) every 1 min for 60 min. (B) A549/Tax cells were treated with EM-E-11-4 (2.5, 5, and 10  $\mu$ M) and/or 100 nM paclitaxel for 48 h. Then, the soluble tubulin and insoluble tubulin were isolated, and the levels of  $\alpha$ -tubulin were determined by Western blot analysis. (C) HeLa/ $\beta$ III cells were treated with EM-E-11-4 (2.5, 5, and 10  $\mu$ M) and/or 100 nM paclitaxel for 48 h. Then, the soluble tubulin and insoluble tubulin were isolated, and the levels of  $\alpha$ -tubulin were determined by Western blot analysis. Columns represent the means $\pm$ SD values for protein levels obtained from three individual experiments. \*  $p < 0.05$  and \*\*  $p < 0.01$  vs. Soluble control (A: paclitaxel 100 nM; B: paclitaxel 20 nM), #  $p < 0.05$  and ##  $p < 0.01$  vs. Insoluble control (A: paclitaxel 100 nM; B: paclitaxel 20 nM).

## Molecular docking analysis of EM-E-11-4 to determine the activity capacity of TUBB3 and P-gp

We investigated the interactions between EM-E-11-4 and TUBB3 or P-gp by molecular docking analysis. The docking results revealed that EM-E-11-4 forms six hydrogen bonds with the residues of Ser-138, Leu-139, Asp-177, Glu-181, Asn-204, and Tyr-222 in TUBB3 (Figure 8A). In addition, the hydrophobic group of EM-E-11-4 inserts into a hydrophobic gap and interacts with Cys-12, Ile-16, Leu-207, Leu-225, and Val-229. Similarly, EM-E-11-4 and P-gp form three hydrogen bonds with the residues of Ser-532, Gln-535, and Tyr-1044, and the hydrophobic groups interact with Phe-512, Leu-516, Leu-531, Ile-1050, and Val-1052 (Figure 8B). Furthermore, the mode of binding in both TUBB3 and P-gp show significant similarity. The macrolide was exposed to the solvent region and formed stable hydrogen bonds and hydrophobic interactions. The phenyl group inserts into the deep hydrophobic gap to form stable hydrophobic interactions. To sum up, critical hydrogen bonds and hydrophobic interactions between EM-E-11-4 and TUBB3 or P-gp were predicted by molecular docking, indicating the EM-E-11-4 may serve as a potent agent by simultaneously targeting TUBB3 and P-gp.

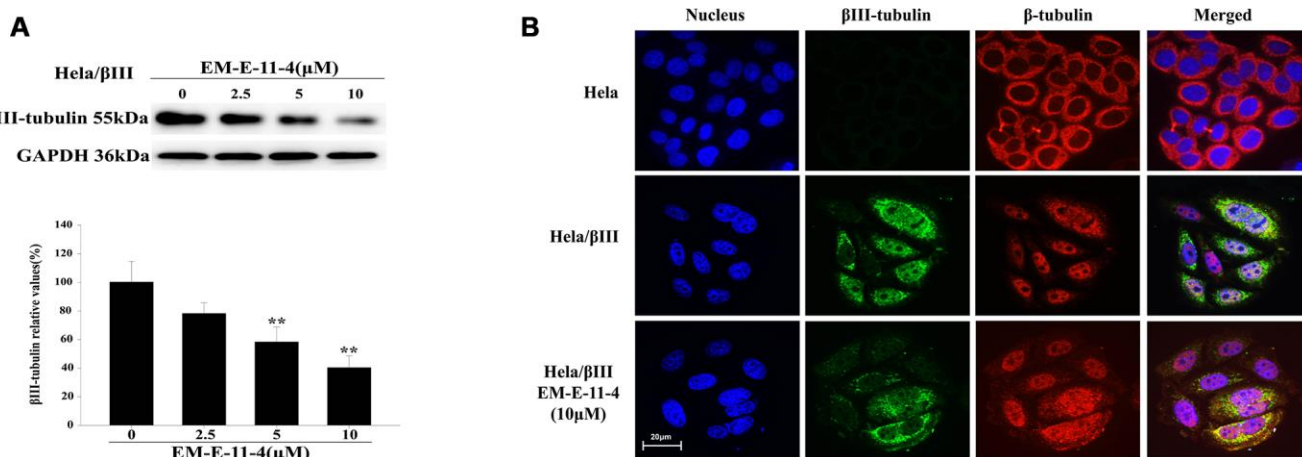
## DISCUSSION

As a first-line drug for various cancer treatment, paclitaxel is frequently used as chemotherapeutic agents. However, paclitaxel has been greatly limited in helping cure cancer by  $\beta$ III-tubulin and P-gp-mediated drug resistance [6–8]. Originated from *Euphorbia*

*micractina*, EM-E-11-4 is a lathyrane-type diterpenoid. Preliminary activity screening results have shown that it could enhance the effects of chemotherapeutic agents, especially paclitaxel, in drug-resistant cancer cells (data not shown). Our study found EM-E-11-4 could markedly enhance paclitaxel cytotoxicity in A549/Tax (P-gp overexpression) and HeLa/ $\beta$ III ( $\beta$ III-tubulin overexpression) cells.

As an ATP-binding cassette transporter, P-gp has been extensively studied for more than 20 years. The overexpression of P-gp is a main mechanism referred to paclitaxel resistance in tumors. It can decrease the accumulation of paclitaxel in cells, which leads to its chemotherapeutic failure. Therefore, inhibition of its expression or its transporting function could reverse P-gp-mediated drug resistance. Many P-gp inhibitors have been developed and dedicated to clinical studies [23], such as verapamil (the 1st-generation nonspecific P-gp inhibitor) [24], valsopodar, biricodar (the 2nd-generation P-gp inhibitor with more potent and more selectivity) [25, 26], and tariquidar (the 3rd-generation P-gp inhibitor with the most potent selectivity) [27].

P-gp inhibition mainly have 3 mechanisms as follows: (a) directly affecting the drug-binding sites of P-gp (competitive inhibitor); (b) affecting ATP binding and hydrolysis, which affects the function of P-gp (noncompetitive inhibitor) [28–29]; (c) affecting the allosteric pocket residues, which inhibits P-gp activity and translocation [30]. EM-E-11-4 was able to up-regulate the accumulation of Flutax-1 and Rhodamine 123 in A549/Tax cells by suppressing the ATPase



**Figure 7. EM-E-11-4 inhibited the expression of  $\beta$ III-tubulin.** (A) HeLa/ $\beta$ III cells were treated with EM-E-11-4 (2.5, 5, and 10  $\mu$ M) for 48 h, and the expression of  $\beta$ III-tubulin was determined by Western blot analysis. Columns represent the means $\pm$ SD values for protein levels obtained from three individual experiments. \*  $p < 0.05$  and \*\*  $p < 0.01$  vs. control (EM-E-11-4 0  $\mu$ M). (B) Immunofluorescence analysis ( $\times 600$ ). Blue: nucleus; green:  $\beta$ III-tubulin; red:  $\beta$ -tubulin. There is no clear green fluorescence in HeLa cells, whereas HeLa/ $\beta$ III cells have a clear green fluorescence. After HeLa/ $\beta$ III cells were treated with EM-E-11-4 48 h, the green fluorescence was weakened.



activity of P-gp rather than changing P-gp expression levels. Based on the mechanisms mentioned above, EM-E-11-4 could be indicated as a noncompetitive inhibitor of P-gp, and thus, it could reverse P-gp-mediated drug resistance in tumors.

Microtubule stabilizing agents (MSA) bind to the tubulin polymer that stabilize MTs and inhibit MT depolymerization, resulting in mitotic arrest (G<sub>2</sub>/M phase arrest) and cell apoptosis [31, 32]. EM-E-11-4 strengthened the effect of paclitaxel-induced tubulin polymerization and promoted tubulin from soluble states to insoluble states. It also increased the binding of paclitaxel to MTs. Therefore, it strengthened the effect of paclitaxel-induced cell arrest in G<sub>2</sub>/M phase and apoptosis in drug-resistant cancer cells. This is a mechanism in which EM-E-11-4 reverses paclitaxel resistance in cancer cells. J. Zhou et al found that the compound EM012 strengthened the anti-proliferative activity of paclitaxel, which originated from its activity of synergistic/additive inhibition of microtubule dynamics [33]. We speculate that EM-E-11-4 may bind near the paclitaxel-binding domain, which leads to a conformation change in the protein. This enhances the ability of paclitaxel to promote tubulin polymerization and bind to MTs.

The relevance of  $\beta$ III-tubulin overexpression with paclitaxel resistance in tumors has been confirmed

within the last 10 years [32]. However, the mechanism of  $\beta$ III-tubulin-mediated tumor drug resistance has not been completely elucidated yet. Previous studies have found that  $\beta$ III-tubulin can enhance the dynamic nature of MTs. Therefore it counteracts the stabilization effect of MT-interacting agents, for example, paclitaxel [34]. Also, the amino acid sequence is different from other  $\beta$ -tubulin isotypes at the paclitaxel-binding domain, which may influence paclitaxel binding to  $\beta$ -tubulin in MTs [35, 36].  $\beta$ III-tubulin was also reported as a factor in cell survival. It was found that  $\beta$ III-tubulin could induce resistance to other drugs in addition to paclitaxel, or it could induce tumor survival in an abnormal environment [37]. At present, there is limited research on  $\beta$ III-tubulin inhibition by the use of siRNAs or miRNAs to silence  $\beta$ III-tubulin at the cellular level, and they are without a breakthrough [37–39]. Until now, there has not been a small molecular compound found that is able to inhibit the expression of  $\beta$ III-tubulin. Our results confirm that EM-E-11-4 inhibits  $\beta$ III-tubulin expression while doesn't affect the other  $\beta$ -tubulin subtypes (data not shown). EM-E-11-4 was indicated as a specific inhibitor of  $\beta$ III-tubulin. This might be the most important reason that EM-E-11-4 can significantly reverse  $\beta$ III-tubulin-mediated paclitaxel resistance in HeLa/ $\beta$ III cells.

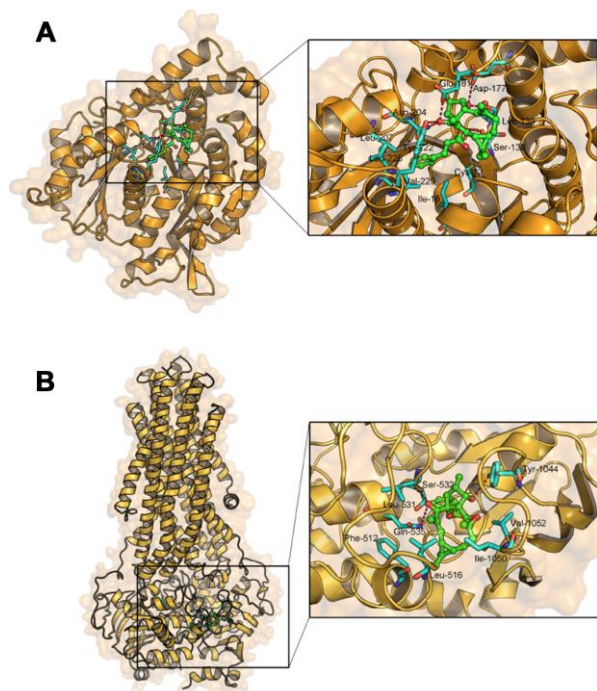
In addition, we investigated the interactions between EM-E-11-4 and TUBB3 or P-gp by molecular docking analysis. Interestingly, the mode of binding in both TUBB3 and P-gp demonstrated significant similarity. Critical hydrogen bonds and hydrophobic interactions between EM-E-11-4 and TUBB3 or P-gp were predicted by molecular docking. A highly similar mode of binding may indicate a similar mechanism of EM-E-11-4 interfering with the function of P-gp and  $\beta$ III-tubulin in paclitaxel resistant tumor cells.

To summarize, our study has demonstrated that EM-E-11-4 could reverse P-gp and  $\beta$ III-tubulin-mediated paclitaxel resistance in tumor cells. It suppressed the ATPase activity of P-gp, which decreases the efflux of paclitaxel by P-gp for the lack of energy. At the same time, it inhibited the expression of  $\beta$ III-tubulin, resulting in a strengthened effect of paclitaxel in HeLa/ $\beta$ III cells. These results suggest that EM-E-11-4 may serve as an efficient MDR reversal agent, particularly for patients with high expression of P-gp or  $\beta$ III-tubulin in tumors.

## MATERIALS AND METHODS

### Materials

Paclitaxel was obtained from Beijing Union Pharmaceutical Factory, with purity over 99%. EM-E-11-4 was isolated from *Euphorbia micractina*, with



**Figure 8.** Molecular docking analysis of EM-E-11-4 to the activity cavity of TUBB3 (A) and P-gp (B).

purity over 99%. Flutax-1 and Flutax-2 were synthesized in our lab, with purity over 99%. The chemical structure of EM-E-11-4 showed in (Figure 1A). Compounds dissolved in dimethyl sulphoxide before use. Rhodamine 123 (Rh123), Verapamil (Vrp), MTT, PI, and RNase A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), DMEM nutrient mixture and RPMI 1640 were obtained from Gibco BRL (Grand Island, NY, USA). TUBB3 siRNA (50-UCUCUUCAGGCCUGACAAU TT-30), P-gp siRNA (50-GCGAAGCAGUGGUUCAG GUTT-30), the negative control (50-UUCUCCGAACG UGUCACGUTT-30) [40] and the Lipofectamine RNAiMAX Reagent were obtained from Invitrogen Trading Co., Ltd. (Shanghai). Apart from the antibodies for  $\beta$ -tubulin,  $\alpha$ -tubulin,  $\beta$ III-tubulin and P-gp which were obtained from Abcam (UK), all other antibodies were obtained from Cell Signaling Technology (USA). From Cytoskeleton (USA), we obtained Tubulin Polymerization Assay Kit (Porcine tubulin fluorescence based, Cat.#BK011P) while Pgp-Glo™ Assay Systems was purchased from Promega (USA). The Annexin V-FITC/PI Apoptosis Kit was purchased from Beyotime Institute of Biotechnology (China). The 4', 6-diamidino-2-phenylindole (DAPI) dihydrochloride nuclear stain, Texas Red® goat anti-rabbit IgG antibody and Alexa Fluor goat anti-mouse IgG antibody were provided from Invitrogen (Carlsbad, CA).

### Cell lines and culture

The human lung cancer cell line A549 and its paclitaxel-resistant counterpart A549/Tax were supplied by Laboratory of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical Collage. Hela and Hela/ $\beta$ III cells were provided from Dr. Richard Ludeña at the University of Texas. A549 and A549/Tax cells were grown in medium RPMI 1640 including 10% FBS, supplemented with 100  $\mu$ g/mL streptomycin and 100 units/mL penicillin. The other cell lines were cultured in DMEM including the same proportion and amount of FBS, streptomycin and penicillin. Drugs (A549/TAX: 10 nM TAX, Hela/ $\beta$ III: 0.5 mg/mL G418 sulfate) were added to the counterpart drug-resistant cell lines, and the medium containing drugs was replaced with the normal medium a week before the experiments. All cells were cultured with 5% CO<sub>2</sub> in air at 37°C.

### MTT assay

Using MTT assay, cell survival rate after EM-E-11-4 and drug treatment was evaluated. Briefly, 5000 cells/well were seeded in 96-well plates, and then 24h later, the cells were treated with the test agent at different concentrations for 3 d. Then, 20  $\mu$ L of 5

mg/mL MTT was added to the wells for 4 h at 37 °C. Next, removed the medium and added 150 $\mu$ L of DMSO to every well. A microplate reader (490 nm) was given to test the absorbance. The IC<sub>50</sub> values for drugs were analyzed by SPSS 17.0.

### siRNA transfection

Cells ( $5 \times 10^4$ ) were seeded in 6-well cell culture plates and cultured for 24 h. Then cells were transfected with siRNAs (TUBB3 siRNA/P-gp siRNA: 100 nM) [40]. After 48 h, used western blotting examined the expression of P-gp and  $\beta$ III-tubulin. siRNA-transfected cells ( $3 \times 10^3$ ) were exposed to different concentrations of compounds. The IC<sub>50</sub> of Taxol with or without EM-E-11-4 in siRNA-transfected cells were analyzed by MTT assay.

### *In vitro* tubulin polymerization assay

Using a Tubulin Polymerization Assay Kit to analyze the capability of EM-E-11-4 and paclitaxel to promote tubulin polymerization. Briefly, tubulin proteins (2  $\mu$ g/ $\mu$ L) were suspended in G-PEM buffer (pH 6.9, 2 mM MgCl<sub>2</sub>, 80 mM PIPES, 1.0 mM GTP, 0.5 mM EGTA and 15% glycerol), and the test agents were added at 4 °C. The sample mixture was transferred to 96-well plates (37°C), and the variations of fluorescence intensity (ex=370 nm, em=445 nm) were measured for 1.5 h.

### Immunofluorescence assay

Hela/ $\beta$ III cells ( $3 \times 10^3$ ) were grown on fibronectin-coated cell culture dishes, and then incubated with or without 10  $\mu$ M EM-E-11-4 for 48 h. Then cells were diluted with PBS, fixed in 4% paraformaldehyde (20 min) and blocked with 5% goat serum in 0.1% Triton X-100 in PBS for 10 min at room temperature. The cells were incubated with anti- $\beta$ III-tubulin and anti- $\beta$ -tubulin antibody (1:100) at 4°C overnight. They were then incubated with the secondary antibody (Texas Red® goat anti-rabbit IgG antibody, Alexa Fluor goat anti-mouse IgG antibody) for 1 h, and then they were incubated with DAPI for 5 min in the dark. Images were examined using a PerkinElmer UltraVIEWVoX system (PerkinElmer Life Sciences Inc., MA, USA).

### P-gp ATPase activity assay

ATPase activity of P-gp was conducted by the Pgp-Glo™ assay system conforming to the instructions. Following incubation with EM-E-11-4 and paclitaxel, the luminometer was used to measure the luminescence of the P-gp ATPase reaction system. The luminescence represents ATP contents, which are negatively related to

the P-gp ATPase activity. This is indicative of the capability of P-gp-mediated transport.

### **The accumulation of Flutax-1**

Flutax-1 is a taxane tagged with a fluorescent group, and it is a fluorescent probe that can selectively combined in taxol-binding site of  $\beta$ -tubulin. Cells ( $5 \times 10^5$ ) were seeded in 6-well plates. Incubation with Flutax-1 and compounds for 3 h, cells were then diluted once with PBS, then observed with a fluorescence microscope.

### **The accumulation of Rhodamine 123 (Rh123)**

Using flow cytometry (Sigma, USA) determined the fluorescence intensity of Rh123 in cells. Briefly, cells ( $5 \times 10^5$ ) were seeded in a 6-well plate and incubated with compounds for 30 min. The cells were harvested, then detected the fluorescence intensity of Rh123 in cells ( $\text{ex}=475\text{nm}$ ,  $\text{em}=525\text{nm}$ ) by utilizing flow cytometry.

### **Analysis of cell cycle**

Flow cytometry was used to detect cell cycle distribution. Cells ( $5 \times 10^5$ ) were seeded in 6-well plates and incubated with compounds for 1 d before the cells were extracted and immersed in 70% ethanol. And cells were incubated with RNase A solution (50  $\mu\text{g}/\text{mL}$ ) and stained with propidium iodide (50  $\mu\text{g}/\text{mL}$ ) for 30 min in the dark. The samples were examined with flow cytometry.

### **Apoptosis assay**

The Annexin V-FITC Apoptosis Detection Kit was used for quantitative determination of apoptotic cells. After incubation with the test agents for 48 h, cells were collected, washed with PBS, and then suspended in 100  $\mu\text{L}$  of binding buffer. A total of 5  $\mu\text{L}$  of Annexin V-FITC was added to the suspension and incubated for 10 min at 25°C. Then, 10  $\mu\text{L}$  of PI was added to the cells and incubated for another 15 min in the dark. Then, 400  $\mu\text{L}$  binding buffer was added prior to analysis by flow cytometry. Early apoptosis is defined the cells in the second phase; Late apoptosis is defined the cells in the third phase.

### **Cell tubulin polymerization assay [41]**

Soluble (cytosolic) and insoluble (cytoskeletal) tubulin from cell lysates were separated as previously described. Briefly, A549/Tax and HeLa/ $\beta$ III cells were incubated with EM-E-11-4 (2.5, 5, 10  $\mu\text{M}$ ) and paclitaxel (A549/Tax: 100 nM; HeLa/ $\beta$ III: 20 nM) for 48 h. Subsequently, cells were lysed at 37°C with 150  $\mu\text{L}$

of hypotonic buffer [41]. The fractions were separated by centrifugation at a speed of 12,000 rpm/20 min at 25°C. The supernatant fraction inclusive of soluble tubulin was transferred to another tube. The precipitation fraction inclusive of insoluble tubulin resuspended in the same buffer. SDS-PAGE extracted and resolved proteins.

### **The determination of paclitaxel binding constants for MTs**

The binding constants of paclitaxel to the paclitaxel binding site of microtubules (MTs) were assessed as previously mentioned [42]. Briefly, Flutax-2 is a taxane with a fluorescence group that can be used as a fluorescent probe to reversibly bind to a taxol-binding site. When paclitaxel was added to the MTs and Flutax-2 mixture, it competitively binds to the taxol-binding site, which leads to Flutax-2 dissociating from MTs. Free Flutax-2 changes the fluorescence polarization value of the reacting system, which calculates the concentration of binding-paclitaxel. Then, according to the binding constants of Flutax-2 for MTs, the binding constants of paclitaxel were calculated.

### **Western blot analysis**

Cells were treated with test agents and then lysed in denaturing lysis buffer (1 mM  $\text{MgCl}_2$ , 20 mM Tris-HCl PH 6.8, 2 mM EGTA, 2  $\mu\text{g}/\text{mL}$  Pepstatin, 1% NP40, 2  $\mu\text{g}/\text{mL}$  Aprotinin, 2 mM PMSF). The samples were isolated by SDS-PAGE, transferred to PVDF membranes. Then membranes were blocked with 5% BSA in TBST for 60 min at 25°C prior to incubation with antibodies against P-gp,  $\beta$ -actin,  $\beta$ III-tubulin,  $\alpha$ -tubulin, and GAPDH overnight at 4°C. Incubated the membranes with their corresponding secondary antibody for 60 min at 25°C, and they were visualized through enhanced chemiluminescence system (GE Healthcare, USA).

### **Molecular docking analysis**

To probe the interactions between EM-E-11-4 and TUBB3 or P-gp, a molecular docking study was performed using AutoDock (version 4.2.6) [43]. Based on a Lamarckian genetic algorithm, AutoDock package is a flexible docking program for searching the best conformation of the ligand in a macromolecule. The crystal structure of human TUBB3 [44] (PDB code: 5IJ0) and P-gp [45] (PDB code: 6C0V) were derived from the Protein Data Bank. AutoDockTools (version 1.5.6) was utilized for generation of the docking input files [44]. A grid box size of 50  $\times$  50  $\times$  50 points with a 0.375 Å spacing between the grid points was applied. Affinity maps of TUBB3 and P-gp were computed through

AutoGrid. The Lamarckian genetic algorithm (LGA) was directed at determination of EM-E-11-4 and protein interaction. The docking parameters are described below: trials of 100 dockings, and the number of individuals in the population were set as 150 and 250,000 energy evaluations. All other settings were left as they were by default. To analyze the docking results, AutoDockTools version 1.5.6 and PyMol came into use [43, 46].

### Statistical analysis

All data were expressed as the mean  $\pm$  standard deviation (SD) of at least three independently performed experiments. The statistical significance of the differences among three groups was analyzed by one-way analysis of variance (ANOVA), which was followed by a least significant difference post-hoc test to obtain individual P values. The Student's t-test was used to determine differences between the two groups. A value  $p < 0.05$  was considered statistically significant.

### CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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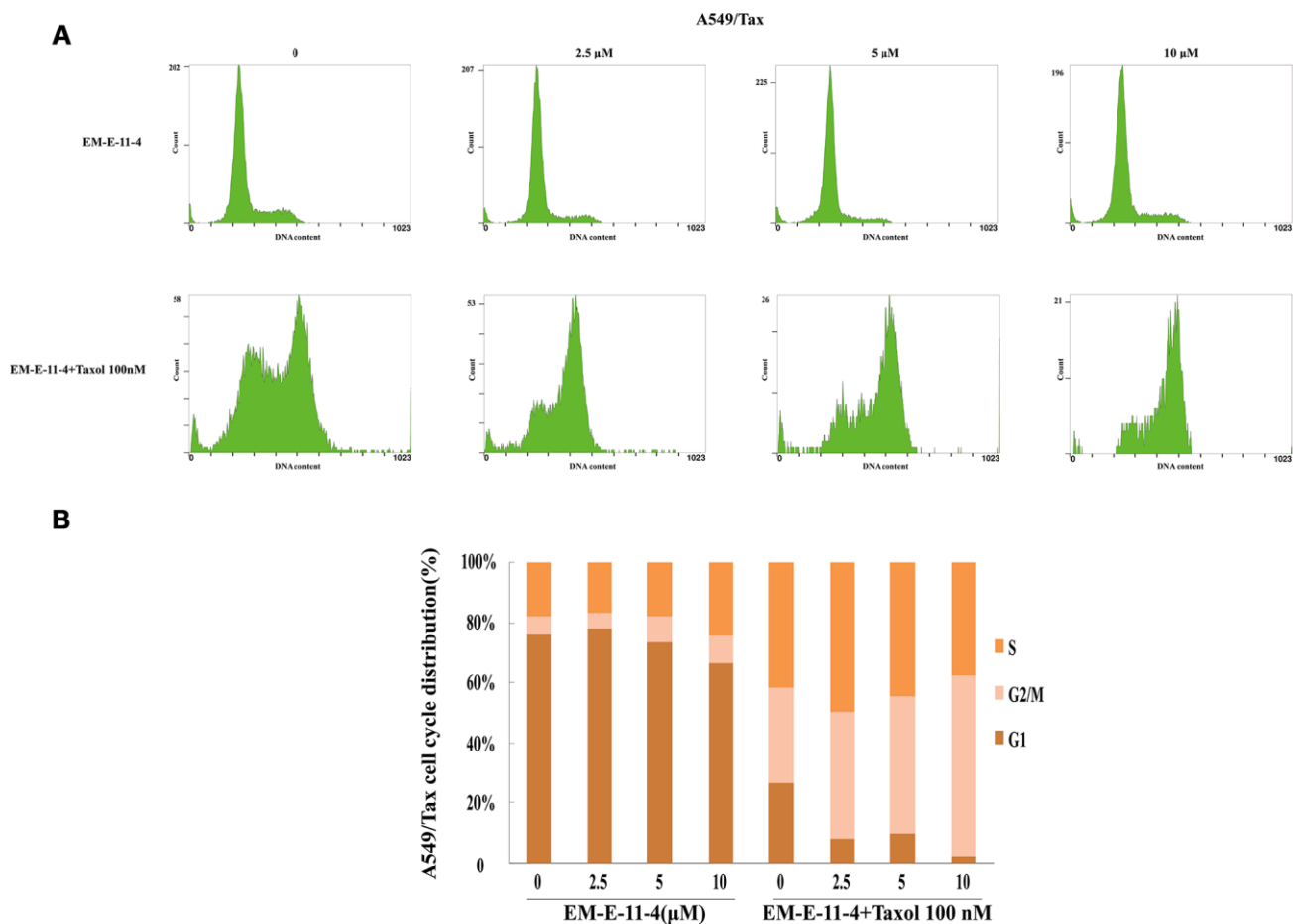
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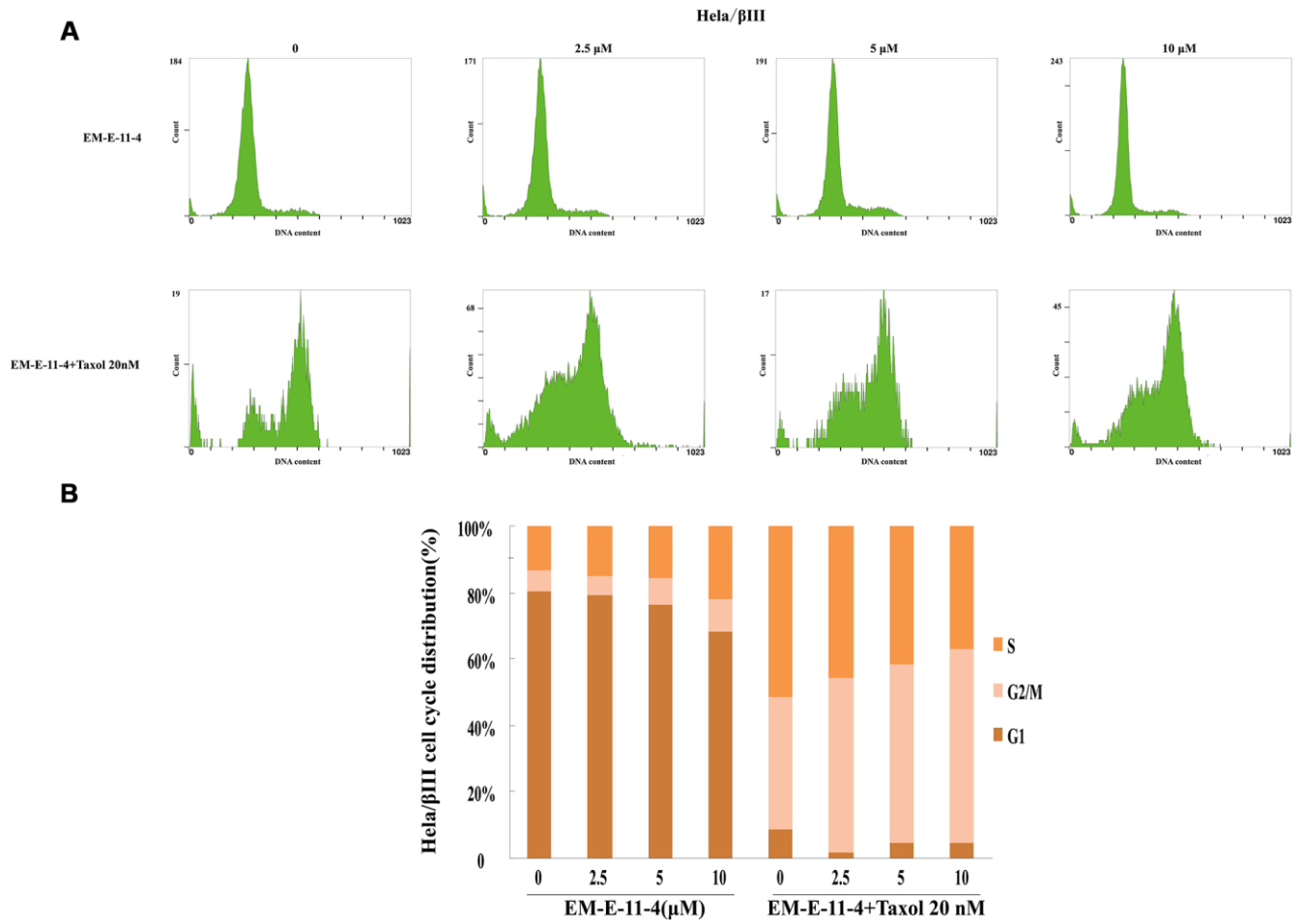
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## SUPPLEMENTARY MATERIALS

### Supplementary Figures



**Supplementary Figure 1. G<sub>2</sub>/M phase arrest induced by EM-E-11-4 and paclitaxel (24 h) in A549/Tax cells. (A)** Cell cycle effects of EM-E-11-4 (2.5, 5, and 10 μM) combined with or without 100 nM paclitaxel in A549/Tax cells. **(B)** The cell cycle distribution of A549/Tax cells.



**Supplementary Figure 2. G<sub>2</sub>/M phase arrest induced by EM-E-11-4 and paclitaxel (24 h) in HeLa/βIII cells. (A)** Cell cycle effects of EM-E-11-4 (2.5, 5, and 10 μM) combined with or without 20 nM paclitaxel in HeLa/βIII cells. **(B)** The cell cycle distribution of HeLa/βIII cells.

## Supplementary Table

**Supplementary Table 1. The binding constants of paclitaxel for MTs (35°C).**

<b>Compound</b>	<b>Binding constants of paclitaxel</b>
/	$0.52 \pm 0.05 \text{ M}^{-1}$
+ EM-E-11-4 $10\mu\text{M}$	$1.59 \pm 0.07 \text{ M}^{-1}$

Data are presented as mean  $\pm$  SD from three independent experiments.