Blockade of GRP78 sensitizes breast cancer cells to microtubules-interfering agents that induce the unfolded protein response

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Abstract

Taxane and vinblastine represent two classes of microtubules-targeted agents for cancer chemotherapy. Although taxol and vinblastine are widely used for cancer treatment, resistance to these agents is frequently encountered in the clinic. An ongoing question has been what mechanisms are involved in the resistance of tumour cells to microtubules-targeted agents or how the clinical effectiveness can be improved. There is increasing evidence that microtubules interact with the endoplasmic reticulum (ER). Here, we have shown that taxol and vinblastine induce multiple arms of the ER stress response, including up-regulation of glucose-regulated protein 78 (GRP78) expression, X-box binding protein 1 splicing and eukaryotic initiation factor 2α phosphorylation. Abrogation of GRP78 induction sensitizes breast cancer cells to taxol and vinblastine. Treatment with (-)-epigallocatechin gallate (EGCG), a known GRP78 inhibitor, synergistically promotes taxol- and vinblastine-induced cell death. GRP78 knockdown or EGCG potentiates taxol- and vinblastine-induced activation of pro-apoptosis arms of the ER stress response, such as JNK phosphorylation, caspase-7 and PARP cleavage. Inhibition of JNK and caspase-7 abrogates EGCG sensitization of breast cancer cells to taxol and vinblastine. We conclude that induction of the unfolded protein response represents a novel mechanism underlying the efficacy and resistance to microtubules-targeted agents. Combination of compounds capable of suppressing GRP78 might be a novel approach for improving the effectiveness of microtubules-targeted chemotherapy.

Keywords: microtubules-targeted agents • glucose-regulated protein 78 • cancer

Introduction

Microtubules are highly dynamic polymers composed of $\alpha\beta$-tubulin dimmers. As an important part of the cytoskeletal framework, microtubules have essential roles in cellular processes, including organelles positioning and transport [1, 2], vesicle trafficking [3] and mitosis [4]. Microtubule-associated proteins (MAPs) and MAPs kinase are essential for the formation of cytoskeletal architecture [5, 6]. Temporal and spatial control of microtubule dynamics is crucial in mitotic spindle assembly, the mitotic checkpoint and chromosome movement [7, 8]. When cell cycle progresses from interphase into mitosis, the cytoskeleton microtubules form properly organized and functional bipolar spindles that attach to chromosomes at the kinetochore, and facilitate the chromosomes segregation at anaphase [7]. It has been postulated that a spindle assembly checkpoint controls the metaphase/anaphase transition to ensure a functional bipolar spindle is formed and the chromosomes are properly aligned before anaphase is initiated [9]. Cell cycle progression of cells that have failed to complete steps during mitosis may be transiently prevented by the spindle checkpoint complex consisting of Bub1, Bub3, BubR1, cdc20 and mad2, which co-operate in transducing the anaphase-delaying signal by inhibiting the ubiquitin ligase activity of anaphase promoting complex [10–13]. Any errors in microtubule attachment to the sister kinetochores can induce aneuploidy. Failure to pass the mitotic checkpoint will lead to cell cycle arrest and apoptotic cell death.

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Microtubules-targeted agents such as taxol and vinblastine are widely used for treatment of many types of cancer, including carcinoma of the breast, ovary, lung, head and neck, prostate, bladder, stomach and oesophagus [14]. The most potent chemotherapeutic mechanism of paclitaxel is kinetic stabilization of spindle microtubule dynamics, which results in mitotic G2/M arrest and apoptosis [15]. In contrast, *Vinca alkaloids* such as vinblastine and vincristine introduce a wedge between two tubulin molecules thereby interfering with microtubule assembly [16]. Just like other classes of small tubulin-binding molecules, tubulin-targeted *Vinca alkaloids* have achieved chemotherapeutic success in selective subsets of patients with cancer. Disruption of microtubule dynamics is responsible for the ability of taxol and vinblastine to inhibit mitotic progression and induce apoptosis.

Resistance to microtubule-targeted therapy is frequently encountered in the clinic. Previous studies have demonstrated that a variety of mechanisms may mediate intrinsic or acquired resistance to taxol-based chemotherapy, including β-tubulin isotypes, PI3K/Akt activation, stathmin and tau overexpression [17–19]. Selective mutations within β-tubulin may interfere with the binding of taxol to its target or alter the microtubule stability. In addition, the spindle assembly checkpoint proteins and dysfunctional regulation of apoptotic signalling pathways contribute to variation in sensitivity to microtubules-targeted drugs [20]. Paclitaxel sensitivity is dependent on a functional spindle assembly checkpoint [21]. The tumour suppressor BRCA1 is linked to mitotic checkpoint through up-regulation of BubR1. BRCA1 down-regulation leads to premature inactivation of spindle checkpoint and confers paclitaxel resistance [22]. Moreover, aurora-A overexpression can override the checkpoint mechanism that monitors mitotic spindle assembly and induce resistance to paclitaxel [23].

The unfolded protein response (UPR) consists of multifaceted signal transduction cascades that are triggered by perturbations in the endoplasmic reticulum (ER) homeostasis. The UPR is not only critical for the development and normal function of secretory cell types, but also important for numerous human diseases such as neurodegenerative diseases, virus infection, diabetes and cancer [24]. Although the UPR is basically a cytoprotective response to ER stress, persistent or unalleviated ER stress will cause cell death. A major UPR regulator is the ER chaperone glucose-regulated protein 78 (GRP78). As a multifunctional protein, GRP78 can interact with transmembrane ER stress sensors such as IRE1, PERK and ATF6 and control their activation; maintain Ca^{2+} homeostasis and target misfolded proteins for proteasomal degradation [25]. Moreover, GRP78 can protect cells from ER-stress-induced apoptosis by preventing the activation of several pro-apoptosis molecules such as caspase-4, caspase-7 and Bik [26–28]. GRP78 is required for ER integrity and ER stress-induced autophagy [29]. Previous studies demonstrate that GRP78 confers resistance to chemotherapeutic drugs such as adriamycin, etoposide, 5-FU and temozolomide [27, 28, 30, 31]. More recently, it has been found that GRP78 confers chemoresistance to tumour-associated endothelial cells [32]. Here, we provide evidence that microtubules-interfering agents induce the UPR in human breast cancer cells. Our results reveal that GRP78 knockdown potentiates the activation of caspase-7 and JNK by taxol and vinblastine thereby sensitizing cancer cells to taxol- and vinblastine-induced cytotoxicity. Furthermore, treatment of breast cancer cells with (−)-epigallocatechin gallate (EGCG), a natural inhibitor of GRP78, sensitizes breast cancer cells to taxol and vinblastine. We have thus identified a novel mechanism of action of microtubules-interfering agents. These results have implications for the understanding of resistance mechanisms as well as the unique efficiency of these drugs.

Materials and methods

Reagents

Paclitaxel (Taxol) and vinblastine were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). EGCG was purchased from MUST Biotech. (Chengdu, China). GRP78 and XBP-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phosphorylated JNK and eIF2α, caspase-7 and PARP antibodies were provided by Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Breast cancer cells were grown in tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2 and were maintained as monolayer cultures in DMEM or RPMI 1640 medium supplemented with 5% foetal bovine serum and 1% penicillin-streptomycin.

Transfection of siRNA

The target sequence used for knockdown of GRP78 was 5′-GGAGCGCAUUGAUACUAGA-3′. The negative control siRNA was purchased from Ribobio Co., Ltd. (Guangzhou, China). The double-stranded siRNA duplex was dissolved in DEPC-treated water. For transfection, 1×10^5 cells were plated into 6-well plates and incubated overnight. LipofecTAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA) was diluted in 250 μl of Opti-MEM I reduced serum medium and incubated at room temperature for 5 min. In addition, siRNA duplex was diluted in 250 μl of Opti-MEM I reduced serum medium and mixed with the pre-diluted LipofecTAMINE 2000. The mixture was incubated at room temperature for 20 min. and 50 nmol/l of siRNA was added into each well and incubated at 37°C.

Western blot

Cells were washed twice with phosphate buffered saline and harvested with cold RIPA lysis buffer containing protease inhibitors (PMSF 1 mmol/l and leupeptin 0.1 g/l). Cell lysates were collected from culture plates using a rubber policeman, and protein collected by centrifugation.

Protein concentrations were determined by BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Forty micrograms of total protein were boiled in 2× loading buffer (0.1 M Tris-Cl, pH 6.8, 4% SDS, 0.2% bromophenyl blue, 20% glycerol) for 10 min., then loaded into Tris-HCl-polyacrylamide gels and transferred electrophoretically to Immobilon-P membrane (Millipore Corporation, Billerica, MA, USA). Membranes were incubated with primary antibodies and appropriate HRP-secondary antibodies. Membranes were additionally probed with an antibody against actin (Santa Cruz Biotechnology) to normalize loading of protein among samples. The secondary antibodies were detected by chemiluminescent agents (Pierce Biotechnology).

Colony formation assay

For GRP78 knockdown experiments, the cells were seeded in 6-well plates at 5000 cells per well. The next day, siCtrl or siGRP78 were transfected. Forty-eight hours later, the cells were treated with or without 0.5 μM taxol and 0.1 μM vinblastine for 6 hrs, and were allowed to form colonies for 14 days. For EGCG experiments, the cells were seeded in 6-well plates at 5000 cells per well. The next day, the cells were treated with or without 10 μM EGCG, 0.5 μM taxol or 0.1 μM vinblastine. Twenty-four hours later, the cells were grown in the absence of drug and were allowed to form colonies for 14 days. The colonies were stained with 1% methylene blue and then counted.

Cell apoptosis assay

Cell apoptosis was assessed by Hoechst 33342 (Sigma-Aldrich, Inc.) staining. Briefly, replicate cultures of 1×10^6 cells per well were plated in 24-well plate. The cells were treated with or without 10 μM EGCG, 0.5 μM taxol or 0.1 μM vinblastine. After a change of fresh medium 24 hrs later, the cells were incubated with 5 μl of Hoechst 33342 solution per well at 37°C for 10 min., followed by examination under a fluorescence microscope. Strong fluorescence or fragmented nuclei can be observed in the nuclei of apoptotic cells, whereas weak fluorescence was observed in live cells. Quantification of apoptotic cells was performed by taking the images in random fields and counting at least 200 cells in four random fields in each well.

WST1 assay

MCF-7 cells were plated in 96-well plates at 5000 cells per well. The next day, cells were treated with or without EGCG, taxol, vinblastine, SP600125 and Ac-DEVD-CHO in four replicates. After 48 hrs, the cytotoxity was assessed by incubating cells with WST1 reagent (Roche, Indianapolis, IN, USA) for 2 hrs and measuring the absorbance at 450 nm, and at 630 nm as reference, with a microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical analysis

One-way ANOVA with least significant difference *post hoc* test was used to test for the differences in cell viability and colony survival rate. All statistical tests were two-tailed, and difference to be considered to be statistically significant when $P < 0.05$.

Results

Taxol and vinblastine induce the unfolded protein response

Both taxol and vinblastine are widely used as broad cytotoxic drugs. To determine whether taxol and vinblastine would impinge on ER stress or the UPR, the effect of taxol and vinblastine on GRP78 expression in cancer cells were examined. First, ZR75–30 cells were treated with different dose of taxol and vinblastine for 24 hrs, and then subjected to Western blot analysis. The results revealed that GRP78 expression was stimulated by both taxol and vinblastine (Fig. 1A). Similar effects were observed in another two breast cancer cell lines, T47D and MCF-7 (Fig. 1B and C). The up-regulation of GRP78 could be detected after treatment of MCF-7 cells with 0.5 μM taxol and 0.1 μM vinblastine for 8 hrs (Fig. 1D).

Next, we investigated the effects of taxol and vinblastine on other ER stress responsive proteins including spliced XBP-1 and phosphorylated eIF2α. Treatment of MCF-7 cells with taxol or vinblastine for 6 hrs promoted XBP-1 splicing (Fig. 2A). During the UPR, a transient translation arrest is induced upon phosphorylation of eIF2α by PERK. The phosphorylation of eIF2α was observed after treatment of breast cancer cells with taxol or vinblastine for 4 hrs (Fig. 2B). Thus, taxol and vinblastine are identified as novel inducers of the UPR.

To determine whether taxol and vinblastine induce the pro-apoptotic components of the UPR, MCF-7 cells were treated with different doses of taxol and vinblastine for 24 hrs, followed by Western blot analysis of JNK phosphorylation, caspase-7 and PARP cleavage. Both taxol and vinblastine induced JNK phosphorylation, caspase-7 and PARP cleavage in a dose-dependent manner, while treatment of MCF-7 cells with 0.5 μM taxol and 0.1 μM vinbastine had little effect on JNK phosphorylation, caspase-7 and PARP cleavage (Fig. 2C).

To determine whether the activation of JNK and caspase-7 contributes to taxol- and vinblastine-induced cell death, the effect of JNK inhibitor and caspase-7 inhibitor on taxol- and vinblastine-induced cytotoxity was determined by WST1 assays. MCF-7 cells were treated with or without 5 μM taxol, 1 μM vinblastine, 10 μM SP600125 or 50 μM Ac-DEVD-CHO. Ac-DEVD-CHO is a caspase-3/7 inhibitor. Because caspase-3 is absent in MCF-7 cells, Ac-DEVD-CHO acts as caspase-7 inhibitor in MCF-7 cells. Treatment of MCF-7 cells with JNK inhibitor partially blocked taxol- and vinblastine-induced cell death (Fig. 3). Also, treatment with caspase-7 inhibitor partially compromised taxol- and vinblastine-induced cell death (Fig. 3). These data imply that the activation of pro-apoptotic arms of the UPR may contribute to taxol- and vinblastine-induced cell death.

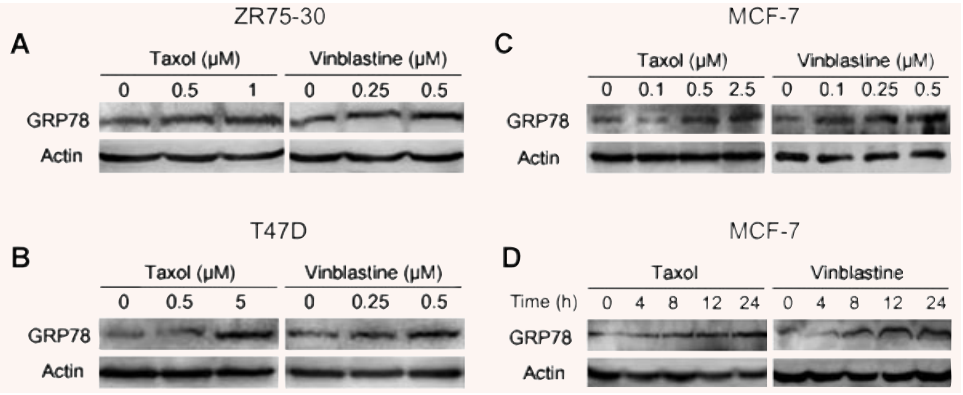

Fig. 1 Taxol and vinblastine induce GRP78 expression in breast cancer cells. (**A**) ZR-75–30 cells were treated with taxol and vinblastine for 24 hrs. Cell lysates were harvested and subjected to Western blot analysis with anti-GRP78 antibody. β-actin was probed as a loading control. (**B**) Western blot analysis of GRP78 expression in response to taxol and vinblastine treatments in T47D cells. (**C**) Western blot analysis of GRP78 expression in response to taxol and vinblastine treatments in MCF-7 cells. (**D**) MCF-7 cells were treated with 0.5 μM taxol and 0.1 μM vinblastine for indicated hours. Cell lysates were harvested and subjected to Western blot analysis with anti-GRP78 antibody.

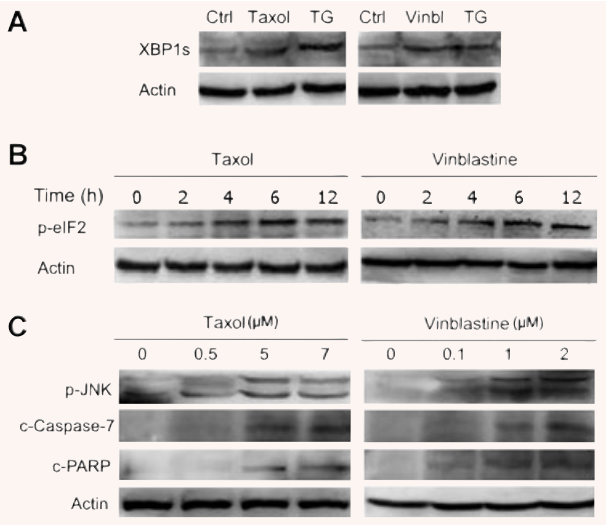

Fig. 2 Taxol and vinblastine induce XBP-1 splicing, eIF2α phosphorylation, JNK phosphorylation and caspase-7 cleavage in breast cancer cells. (**A**) MCF-7 cells were treated with 0.5 μM taxol, 0.1 μM vinblastine and 300 nM TG for 6 hrs. Total proteins were harvested and subjected to Western blot analysis of the spliced XBP-1 (XBP1s). (**B**) MCF-7 cells were treated with 0.5 μM taxol and 0.1 μM vinblastine for indicated periods. Total proteins were harvested and subjected to Western blot analysis of the phosphorylated eIF2α. (**C**) MCF-7 cells were treated with indicated doses of taxol and vinblastine for 24 hrs. Total proteins were harvested and subjected to Western blot analysis of the phosphorylated JNK, cleaved caspase-7 and PARP.

Suppression of GRP78 potentiates the activation of JNK and caspase-7 by taxol and vinblastine

Because GRP78 represents a pro-survival arm in the UPR, down-regulation of GRP78 may break the balance of pro-survival signals and pro-apoptosis molecules. To investigate whether GRP78 down-regulation by siRNA potentiates the activation of pro-apoptotic components by microtubules-targeted agents, the effects of GRP78 knockdown on JNK phosphorylation, caspase-7 activation and PARP cleavage were examined. EGCG alone did not induce JNK phosphorylation and caspase-7 cleavage. Treatment with 0.5 μM taxol or 0.1 μM vinblastine alone significantly up-regulated GRP78 expression, weakly induced JNK phosphorylation, but had little effects on caspase-7 and PARP cleavage. In contrast, GRP78 knockdown resulted in dramatic increase in taxol- and vinblastine-induced JNK phosphorylation, caspase-7 and PARP cleavage (Fig. 4A). These data indicate that blockade of GRP78 induction can potentiate the activation of pro-apoptosis molecules by both taxol and vinblastine.

Previous studies have identified EGCG, the major component of green tea, as a natural compound that directly interacts with the ATP-binding domain of GRP78, blocks its interaction with procaspase-7 and suppresses the protective function of GRP78 [33]. To investigate whether EGCG can enhance taxol- and vinblastine-induced JNK phosphorylation, caspase-7 activation and PARP cleavage, cells were treated with EGCG, taxol or both. Treatment with EGCG alone did not induce JNK phosphorylation, caspase-7 and PARP cleavage. However, combination of EGCG and taxol resulted in much higher levels of phosphorylated JNK, cleaved caspase-7 and PARP than that in cells treated with taxol alone (Fig. 4B). Similar effects were observed in cells treated with EGCG and vinblastine (Fig. 4B). These results demonstrated that EGCG could potentiate the pro-apoptotic signals induced by microtubules-interfering agents.

Abrogation of GRP78 induction sensitizes breast cancer cells to taxol and vinblastine

To test whether breast cancer cells depend on GRP78 for protection against microtubules-interfering agents-mediated cell death,

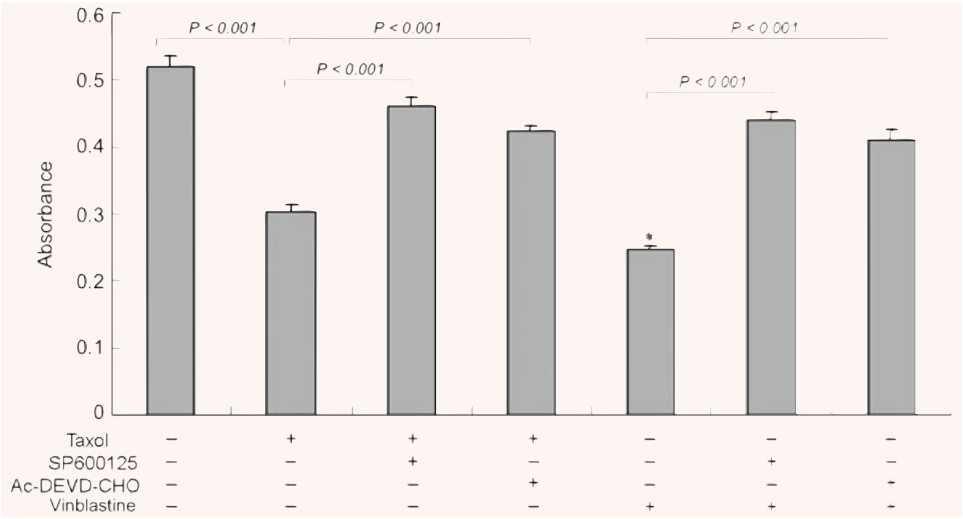

Fig. 3 Inhibition of JNK and caspase-7 protects breast cancer cells from taxol and vinblastine. MCF-7 cells were plated in 96-well plates at 5000 cells per well. The next day, the cells were treated with 5 μM taxol, 1 μM vinblastine, 10 μM SP600125 or 50 μM Ac-DEVD-CHO. Control cells were treated with the vehicle. Forty-eight hours later, the cytotoxicity was assessed by incubating cells with WST1 reagent for 2 hrs and measuring the absorbance at 450 nm, and at 630 nm as reference, with a microplate reader. These experiments were carried out with four replicates. *Bars*, S.E. *, $P < 0.001$, compared with control. A representative of three experiments is shown.

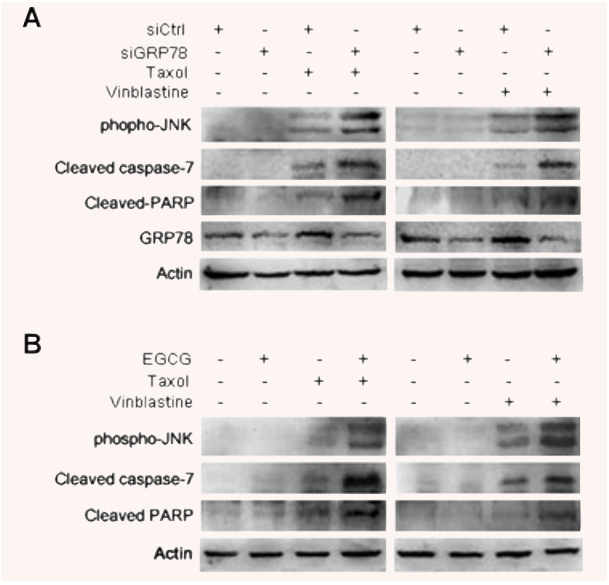

Fig. 4 GRP78 knockdown and EGCG potentiate JNK phosphorylation, caspase-7 and PARP cleavage after taxol and vinblastine treatments. (**A**) MCF-7 cells were transfected with negative control siRNA (siCtrl) or siRNA against GRP78 (siGRP78). Forty-eight hours later, the cells were treated with 0.5 μM taxol or 0.1 μM vinblastine for further 24 hrs. Total proteins were harvested and subjected to Western blot analysis of phosphorylated JNK, cleaved caspase-7 and PARP. (**B**) MCF-7 cells were treated with 10 μM EGCG, 0.5 μM taxol or 0.1 μM vinblastine for 24 hrs. Total proteins were harvested and subjected to Western blot analysis of phosphorylated JNK, cleaved caspase-7 and PARP.

breast cancer cells were transfected with siRNA against GRP78 (siGRP78) or the negative control siRNA (siCtrl), followed by treatment with or without taxol and vinblastine. Colony survival assay was done to evaluate the drug sensitivity of the siGRP78-transfected cells. Western blot analysis verified that siGRP78 was highly effective in suppressing GRP78 expression (Fig. 5). The results demonstrated that abrogation of GRP78 induction led to a significant decrease in colony survival for taxol- or vinblastine-treated cells (Fig. 5). These data indicate that suppression of GRP78 expression may lead to increased sensitivity of breast cancer cells to taxol and vinblastine.

The above-mentioned results imply that down-regulation of GRP78 by compounds directed against GRP78 expression or activity could lead to increased sensitivity of breast cancer cells to microtubules-targeted agents. To determine whether the combination of EGCG and taxol or vinblastine can synergistically promote breast cancer cells death, the effect of EGCG, taxol or vinblastine alone and combination on cell survival was examined by colony formation assay. The results demonstrated that treatment of cells with EGCG in combination with taxol or vinblastine led to a dramatic decrease in cell survival compared to treatment with taxol or vinblastine alone (Fig. 6).

To determine whether apoptosis is responsible for the decreased viability in cells that are treated with EGCG and microtubules-targeted agents, the effect of EGCG, taxol or vinblastine alone and combination on cell apoptosis was examined by Hoechst 33342 staining. The results demonstrated that combination of EGCG and taxol or vinblastine led to significant increase in apoptosis compared to treatment with taxol or vinblastine alone (Fig. 7).

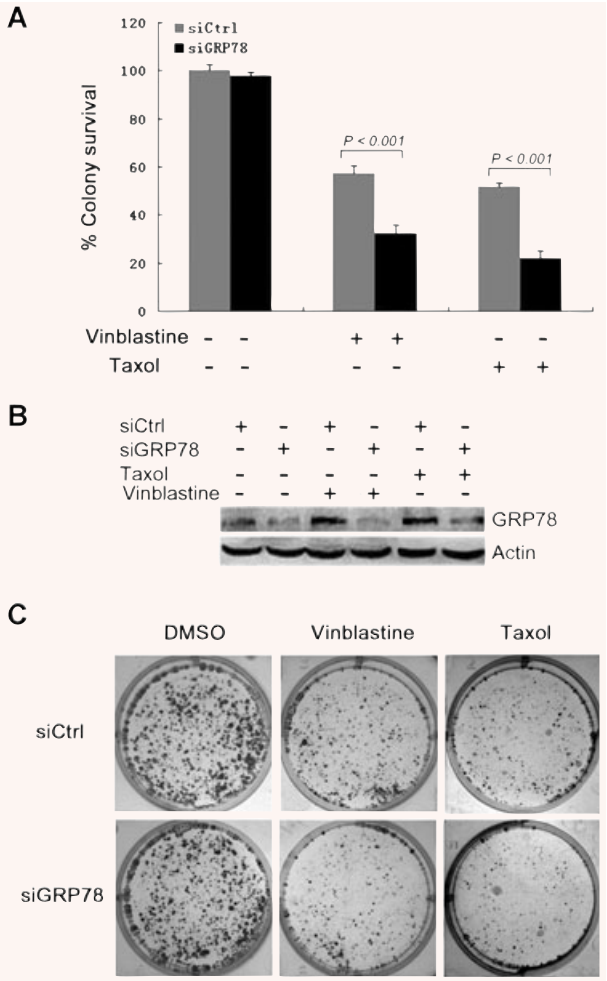

Fig. 5 GRP78 knockdown sensitizes breast cancer cells to taxol and vinblastine. (**A**) MCF-7 cells were seeded in a 6-well plate at 5000 cells per well. The next day, cells were transfected with siRNA against GRP78 (siGRP78) or negative control siRNA (siCtrl). Forty-eight hours after transfection, the cells were treated with 0.5 μM taxol or 0.1 μM vinblastine for 6 hrs, and were allowed to form colonies for 14 days. The percentage of colony survival was plotted. Standard error represents three experiments. **(B)** In parallel, cells transfected with siRNA and treated with or without taxol and vinblastine were harvested. The protein lysates were analyzed by Western blot with antibodies against GRP78 and actin. (**C**) Representative pictures of the colonies from all treatment groups.

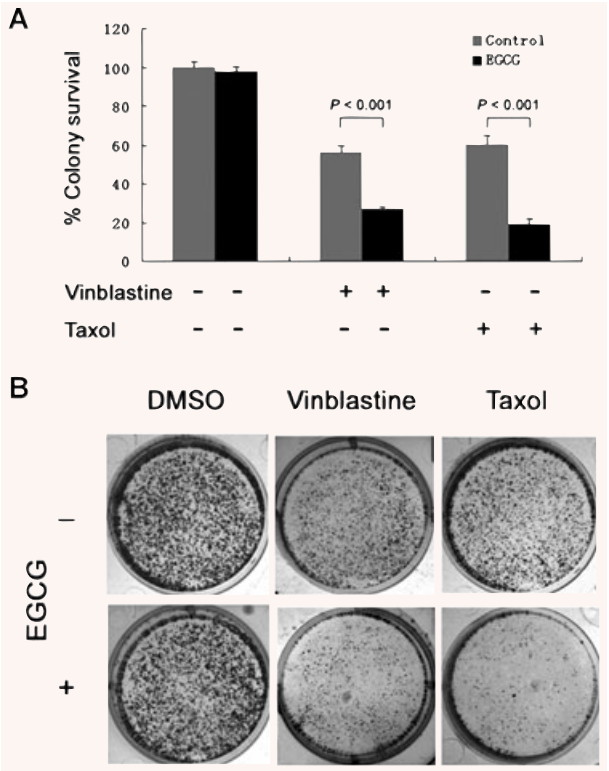

Fig. 6 EGCG sensitizes breast cancer cells to taxol and vinblastine. (**A**) MCF-7 cells were seeded in a 6-well plate at 5000 cells per well. The next day, the cells were treated with 0.5 μM taxol or 0.1 μM vinblastine for 6 hrs and with EGCG for 24 hrs. The cells were allowed to form colonies for 14 days in the absence of drugs. The percentage of colony survival was plotted. Standard error represents three experiments. (**B**) Representative pictures of the colonies from all treatment groups.

Inhibition of JNK and caspase-7 abrogates the sensitization of breast cancer cells to microtubules-targeted agents by EGCG

To determine whether the potentiation of JNK phosphorylation and caspase-7 cleavage by EGCG is responsible for EGCG sensitization of breast cancer cells to taxol and vinblastine, the effect of JNK inhibitor and caspase-7 inhibitor on EGCG-, taxol- and vinblastine-induced cytotoxity was determined by WST1 assays. MCF-7 cells were treated with or without 10 μM EGCG, 0.5 μM taxol, 0.25 μM vinblastine, 10 μM SP600125 or 50 μM Ac-DEVD-CHO for 48 hrs. Treatment of MCF-7 cells with EGCG alone had little effect on cell survival, whereas treatment with 0.5 μM taxol or 0.25 μM vinblastine had modest but significant cytotoxic effect (Fig. 8). Treatment with JNK inhibitor partially blocked taxol- and vinblastine-induced cell death, whereas caspase-7 inhibitor had little effect on taxol- and vinblastine-induced cell death (Fig. 8). These data suggest that JNK, but not caspase-7, contributes to cell death induced by relatively low dose of taxol and vinblastine. Treatment with EGCG sensitized breast cancer cells to both taxol and vinblastine. Strikingly, both JNK inhibitor and caspase-7 inhibitor blocked EGCG sensitization of MCF-7 cells to taxol- and vinblastine-induced cytotoxity (Fig. 8). These data suggest that the potentiation of taxol- and vinblastine-induced JNK and caspase-7 activation by EGCG is involved in its sensitization of cancer cells to taxol and vinblastine.

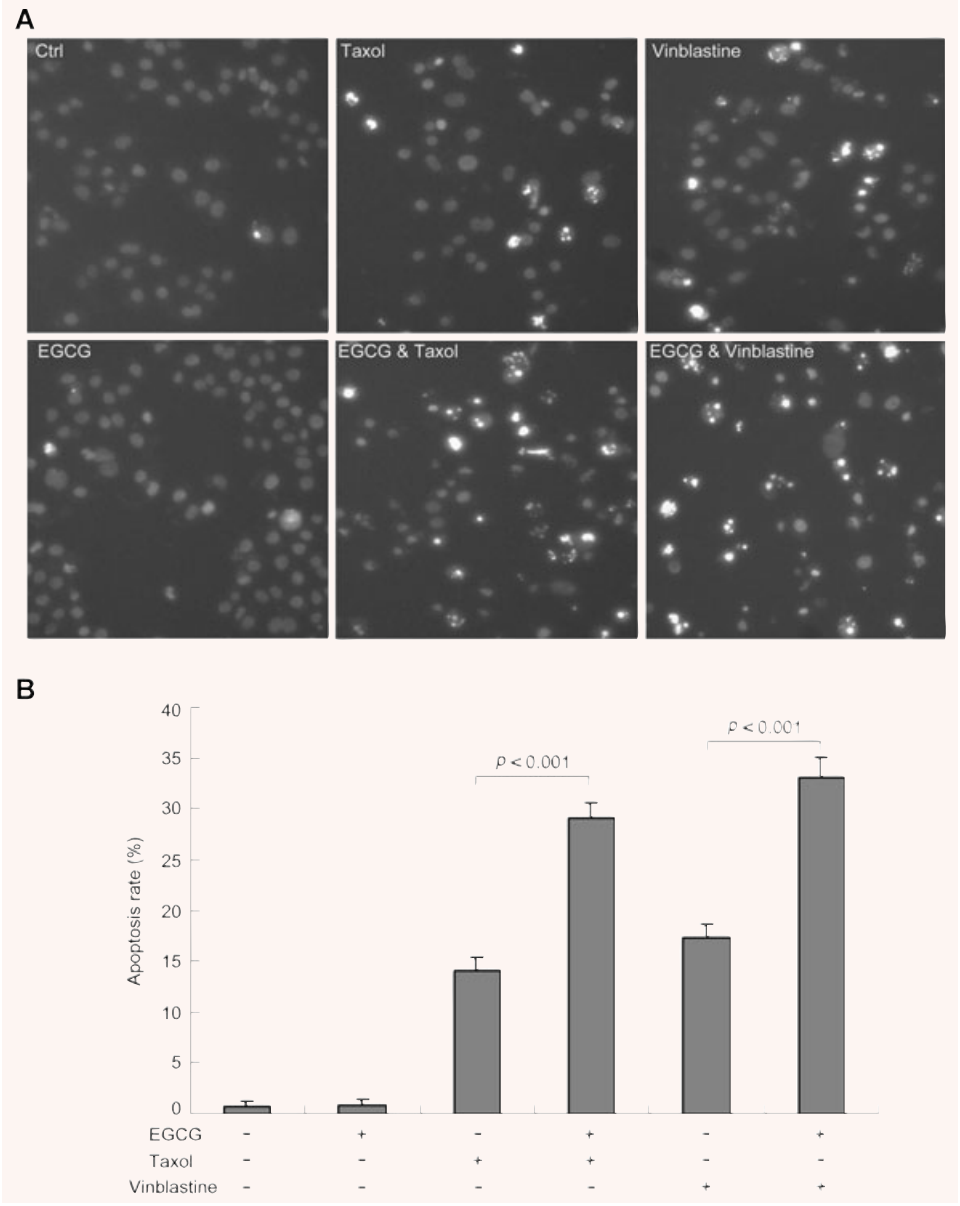

Fig. 7 Treatment with EGCG potentiates taxol- and vinblastine-induced apoptosis. (**A**) MCF-7 cells were treated with or without 10 μM EGCG, 0.5 μM taxol or 0.1 μM vinblastine for 24 hrs, stained with Hoechst 33342, and examined under fluorescent microscopy. Live cells show pale nuclear staining pattern. The apoptotic cells show strong Hoechst 33342^{+} staining or fragmented nuclei. (**B**) The apoptotic cells in four randomized fields were counted. The percent of apoptotic MCF-7 cells was plotted. The columns represent the mean of triplicate wells, and the bars represent the S.E.

Discussion

Microtubules-interfering agents are attractive in cancer chemotherapy. Natural products such as taxol, a compound from pacific yew and vinblastine, a *Vinka alkoloids*, have been successfully used in the clinic for decades. In this study, we have made several new observations, which may have important implication in understanding the mechanisms of action and resistance. Our results showed that both taxol and vinblastine can induce ER stress response, including the up-regulation of ER chaperone GRP78, induction of XBP-1 splicing and phosphorylation of eIF2α.

An important question arises as to how taxol and vinblastine induce ER stress response. Both taxol and vinblastine can interfere with microtubules dynamics in different ways. The mechanism of ER stress response to microtubules-targeted agents is not clear and is likely to be complex. A connection of microtubules to ER remodelling is just emerging. ER tubules were often aligned with microtubules [2, 34]. Both ER and microtubules retract in taxol-treated cells [2]. Moreover, it has been revealed that ER extension occurs through a process whereby an ER tubule attaches to and elongates together with a growing microtubule. Polymerization of individual microtubules and extension of individual

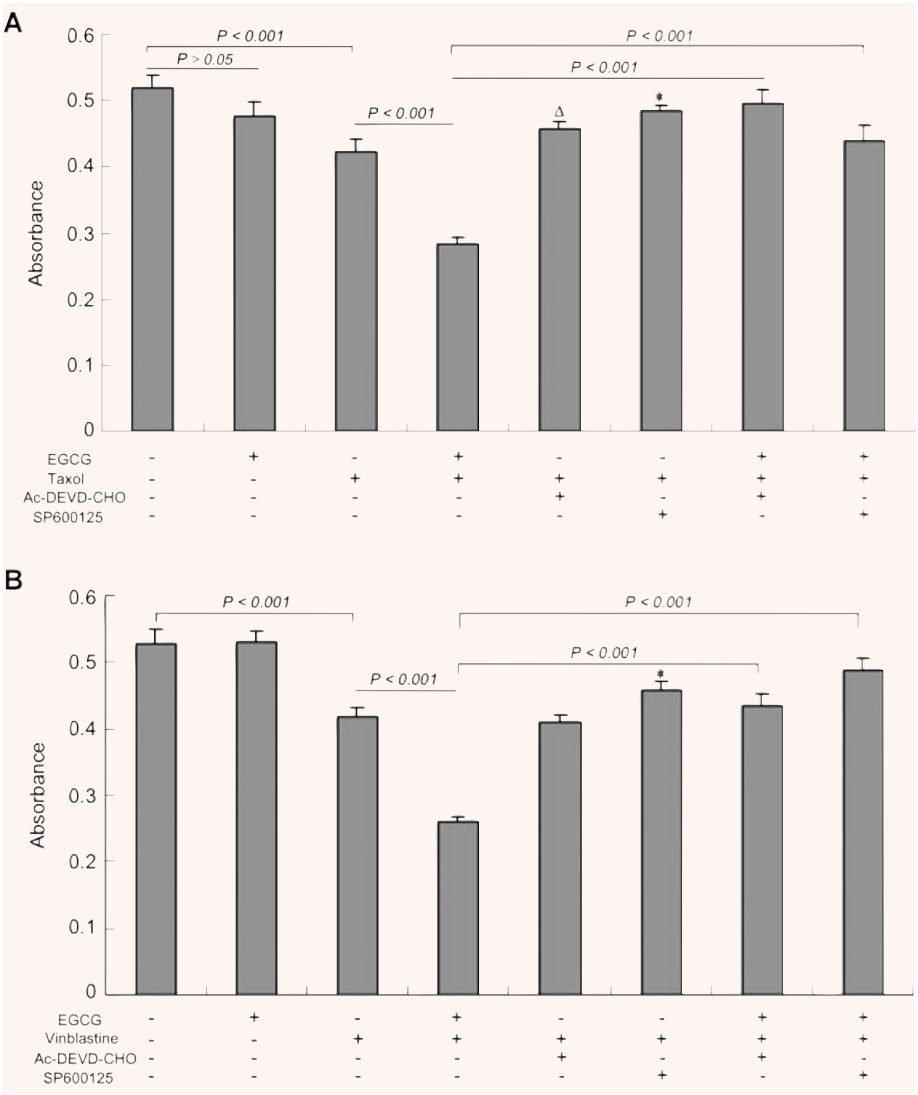

Fig. 8 Inhibition of JNK and caspase-7 abrogates EGCG sensitization of breast cancer cells to taxol and vinblastine. (**A**) MCF-7 cells were plated in 96-well plates at 5000 cells per well. The next day, the cells were treated with 0.5 μM taxol, 10 μM SP600125 or 50 μM Ac-DEVD-CHO. Control cells were treated with the vehicle. Forty-eight hours later, the cytotoxicity was assessed by incubating cells with WST1 reagent for 2 hrs and measuring the absorbance at 450 nm, and at 630 nm as reference, with a microplate reader. These experiments were carried out with four replicates. *Bars*, S.E. Δ, $P > 0.05$; *, $P < 0.02$, compared with taxol-treated cells. (**B**) MCF-7 cells were plated in 96-well plates at 5000 cells per well. The next day, the cells were treated with 0.25 μM vinblastine, 10 μM SP600125 or 50 μM Ac-DEVD-CHO. Control cells were treated with the vehicle. Forty-eight hours later, the cytotoxity was assessed by incubating cells with WST1 reagent for 2 hrs and measuring the absorbance at 450 nm, and at 630 nm as reference, with a microplate reader. These experiments were carried out with four replicates. *Bars*, S.E. *, $P < 0.01$, compared with vinblastine-treated cells. A representative of three experiments is shown.

ER tubules occur together. The intact microtubule system is required to maintain the extended state of the entire ER network [34]. Previous studies have suggested that the absence of microtubules may lead to the failure in ER extensions in nocodazole-treated cells, and ER extensions may be hampered in taxol-treated cells because taxol-stabilized microtubules move retrogradely and there is no polymerization of new microtubule tracks for ER elongation [2]. The integral membrane proteins such as P63 may mediate the interaction between microtubules and the ER [35]. These evidence may help to understand how microtubules-interfering agents can disrupt ER remodelling or homeostasis and induce the UPR.

The UPR is associated with a variety of physiological and developmental processes as well as diseases. Although the UPR is regarded as a cytoprotective process, prolonged or unalleviated ER stress may activate multiple pro-apoptosis signalling pathways. However, tumour cells are generally more resistant to ER stress-induced apoptosis compared with un-transformed cells. It is known that some of the pro-apoptotic components in the UPR are suppressed in cancer cells. The protective elements of the UPR, on the other hand, are enhanced in stress-resistant cancer cells. Some aspects of tumour biology may be altered by UPR activation. Notably, alteration in the environment of the ER and UPR activation may affect the chemosensitivity of tumours. For example, treatment of cancer cells with drugs that induce ER stress renders these cells resistant to topoisomerase II inhibitors but sensitive to TRAIL and cisplatin [36, 37]. Therefore, it appears that activation of the UPR can either synergize with or attenuate

the efficacy of various anticancer drugs, depending on the mechanisms of action of the agents.

The mechanism of resistance to microtubules-interfering agents may be complex. Previous studies reveal that key pro-survival molecules such as Akt, MAPs, mitotic checkpoint proteins and regulators of ceramide metabolism are involved in taxol resistance [19, 38]. Our studies suggest that the protective arms in the UPR are involved in resistance to taxane-based therapy. Overexpression of GRP78 in cancer cells may confer resistance to both taxol and vinblastine. As a major ER chaperone, GRP78 can suppress ER stress-induced apoptosis by interacting with caspase-4, caspase-7 and Bik and inhibiting their activation [26–28]. Our studies reveal that taxol or vinblastine treatment leads to activation of caspase-7, which can be potentiated by knockdown of GRP78. In addition, previous studies have demonstrated that JNK pathway is required for efficient apoptosis induced by taxol and vinblastine [39]. Activation of JNK is also involved in ER stress-induced apoptosis. In this study, we demonstrated that GRP78 knockdown resulted in more significant phosphorylation of JNK by both taxol and vinblastine. Although microtubules-interfering agent as a single reagent has a pro-apoptotic effect, this effect may be antagonized by compensatory cellular mechanisms such as the UPR. Further, colony survival assay revealed that down-regulation of GRP78 sensitized breast cancer cells to both taxol and vinblastine. Taken together, it is obvious from our research that taxol- and vinblastine-induced up-regulation of GRP78 protects cancer cells against cell death induced by these drugs. This is in agreement with a previous finding that GRP78 levels were increased in a taxol resistant MCF-7 subline [40]. Our study demonstrates that the overexpressed GRP78 is not a by-stander, but a contributor to taxol and vinblastine resistance. While this manuscript was in preparation, an independent study demonstrated that ER stress was involved in docetaxel-induced apoptosis in melanoma cells [41]. Because GRP78 overexpression is also linked to the development of resistance to topoisomerase-targeted drugs, it is possible that taxol- and vinblastine-induced GRP78 overexpression would lead to resistance to multiple drugs.

Because GRP78 represents a pro-survival arm in the UPR that is induced by selective chemotherapeutic drugs, abrogation of GRP78 induction may be a strategy to sensitize cancer cells to taxol and vinblastine. Indeed, this study clearly demonstrated that down-regulation of GRP78 by siRNA synergistically promotes taxol- or vinblastine-induced breast cancer cells death. Previous studies have shown that inhibition of GRP78 can sensitize cancer cells to therapeutic agents including VP-16 [27, 42], temozolomide [30], adriamycin–cisplatin [31] and quercetin [43]. Therefore, depletion of GRP78 may be an important adjunct in cancer therapy. Because GRP78 is also expressed at cancer cells surface, it may serve as a target for delivery of chemotherapeutic agents to tumours. A cyclic peptide-taxol conjugate has been shown to target GRP78-expressing cancer cells and selectively kill cancer cells [44]. In addition to regulate cell survival under ER stress, GRP78 have critical roles in tumour proliferation and angiogenesis [45]. Moreover, it has been demonstrated that GRP78 is involved in Akt phosphorylation [46]. Thus, the developments of small molecule inhibitors that specifically suppress GRP78 induction or its activity are justified. Recently, EGCG, a major component of green tea, has been identified as a natural GRP78 inhibitor [33]. Treatment with EGCG sensitizes malignant glioma cells to temozolomide [30]. In this study, we showed that treatment with EGCG had similar effects as GRP78 knockdown. Breast cancer cells treated with the combination of EGCG and taxol or vinblastine exhibited much less survival than cells treated with taxol or vinblastine alone. Altogether, these studies indicate that combination of GRP78 inhibitors may be a novel approach for improving the effectiveness of microtubules-targeted agents.

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References

1. **Rowning BA, Wells J, Wu M, *et al.*** Microtubule-mediated transport of organelles and localization of β-catenin to the future dorsal side of Xenopus eggs. *Proc Natl Acad Sci USA.* 1997; 94: 1224–9.
2. **Terasaki M, Reese TS.** Interactions among endoplasmic reticulum, microtubules, and retrograde movements of the cell surface. *Cell Motil Cytoskel.* 2008; 29: 291–300.
3. **Balasubramanian N, Scott DW, Castle JD, *et al.*** Arf6 and microtubules in adhesion-dependent trafficking of lipid rafts. *Nat Cell Biol.* 2007; 9: 1381–91.
4. **Scholey JM, Rogers GC, Sharp DJ.** Mitosis, microtubules, and the matrix. *J Cell Biol.* 2001; 154: 261–6.
5. **Halpain S, Dehmelt L.** The MAP1 family of microtubule-associated proteins. *Genome Biol.* 2006; 7: 224.
6. **Horne MM, Guadagno TM.** A requirement for MAP kinase in the assembly and maintenance of the mitotic spindle. *J Cell Biol.* 2003; 161: 1021–8.
7. **Gorbsky GJ.** Kinetochores, microtubules and the metaphase checkpoint. *Trends Cell Biol.* 1995; 5: 143–8.
8. **McKim KS, Hawley RS.** Chromosomal control of meiotic cell division. *Science.* 1995; 270: 1595–601.
9. **Kirsch-Volders M, Cundari E, Verdoodt B.** Towards a unifying model for the metaphase/anaphase transition. *Mutagenesis.* 1998; 13: 321–35.
10. **Li X, Nicklas RB.** Mitotic forces control a cell-cycle checkpoint. *Nature.* 1995; 373: 630–2.
11. **Brady DM, Hardwick KG.** Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. *Curr Biol.* 2000; 10: 675–8.

12. **Chen RH.** BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1. *J Cell Biol.* 2002; 158: 487–96.
13. **Sudakin V, Chan GK, Yen TJ.** Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, MAD2. *J Cell Biol.* 2001; 154: 925–36.
14. **Singh P, Rathinasamy K, Mohan R, *et al.*** Microtubule assembly dynamics: an attractive target for anticancer drugs. *IUBMB Life.* 2008; 60: 368–75.
15. **Yvon AM, Wadsworth P, Jordan MA.** Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Mol Biol Cell.* 1999; 10: 947–59.
16. **Gigant B, Wang C, Ravelli RB, *et al.*** Structural basis for the regulation of tubulin by vinblastine. *Nature.* 2005; 435: 519–22.
17. **Kavallaris M, Kuo DY, Burkhart CA, *et al.*** Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isotypes. *J Clin Invest.* 1997; 100: 1282–93.
18. **Orr GA, Verdier-Pinard P, McDaid H, *et al.*** Mechanisms of Taxol resistance related to microtubules. *Oncogene.* 2003; 22: 7280–95.
19. **Rouzier R, Rajan R, Wagner P, *et al.*** Microtubule-associated protein tau: a marker of paclitaxel sensitivity in breast cancer. *Proc Natl Acad Sci USA.* 2005; 102: 8315–20.
20. **Swanton C, Marani M, Pardo O, *et al.*** Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. *Cancer Cell.* 2007; 11: 498–512.
21. **Sudo T, Nitta M, Saya H, *et al.*** Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint. *Cancer Res.* 2004; 64: 2502–8.
22. **Chabalier C, Lamare C, Racca C, *et al.*** BRCA1 downregulation leads to premature inactivation of spindle checkpoint and confers paclitaxel resistance. *Cell Cycle.* 2006; 5: 1001–7.
23. **Anand S, Penrhyn-Lowe S, Venkitaraman AR.** AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell.* 2003; 3: 51–62.
24. **Iwakoshi NN, Lee AH, Vallabhajosyula P, *et al.*** Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat Immunol.* 2003; 4: 321–9.
25. **Li J, Lee AS.** Stress induction of GRP78/BiP and its role in cancer. *Curr Mol Med.* 2006; 6: 45–54.
26. **Jiang CC, Chen LH, Gillespie S, *et al.*** Inhibition of MEK sensitizes human melanoma cells to endoplasmic reticulum stress-induced apoptosis. *Cancer Res.* 2007; 67: 9750–61.
27. **Reddy RK, Mao C, Baumeister P, *et al.*** Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. *J Biol Chem.* 2003; 278: 20915–24.
28. **Fu Y, Li J, Lee AS.** GRP78/BiP inhibits endoplasmic reticulum BIK and protects human breast cancer cells against estrogen starvation–induced apoptosis. *Cancer Res.* 2007; 67: 3734–40.
29. **Li J, Ni M, Lee B, *et al.*** The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. *Cell Death Differ.* 2008; 15: 1460–71.
30. **Pyrko P, Schonthal AH, Hofman FM, *et al.*** The unfolded protein response regulator GRP78/Bip as a novel target for increasing chemosensitivity in malignant gliomas. *Cancer Res.* 2007; 67: 9809–16.
31. **Lee AS.** GRP78 induction in cancer: therapeutic and prognostic implications. *Cancer Res.* 2007; 67: 3496–9.
32. **Virrey JJ, Dong D, Stiles C, *et al.*** Stress chaperone GRP78/BiP confers chemoresistance to tumor-associated endothelial cells. *Mol Cancer Res.* 2008; 6: 1268–75.
33. **Ermakova SP, Kang BS, Choi BY, *et al.*** (-)-Epigallocatechin gallate overcomes resistance to etoposide-induced cell death by targeting the molecular chaperone glucose-regulated protein 78. *Cancer Res.* 2006; 66: 9260–9.
34. **Terasaki M, Chen LB, Fujiwara K.** Microtubules and the endoplasmic reticulum are highly interdependent structures. *J Cell Biol.* 1986; 103: 1557–68.
35. **Farah CA, Liazoghli D, Perreault S, *et al.*** Interaction of microtubule-associated protein-2 and p63: a new link between microtubules and rough endoplasmic reticulum membranes in neurons. *J Biol Chem.* 2005; 280: 9439–49.
36. **Ma Y, Hendershot LM.** The role of the unfolded protein response in tumor development: friend or foe? *Nat Rev Cancer.* 2004; 4: 966–77.
37. **Jiang CC, Chen LH, Gillespie S, *et al.*** Tunicamycin sensitizes human melanoma cells to tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis by up-regulation of TRAIL-R2 via the unfolded protein response. *Cancer Res.* 2007; 67: 5880–8.
38. **McGrogan BT, Gilmartin B, Carney DN, *et al.*** Taxanes, microtubules and chemoresistant breast cancer. *Biochim Biophys Acta.* 2008; 785: 96–132.
39. **Fan M, Goodwin ME, Birrer MJ, *et al.*** The c-Jun NH(2)-terminal protein kinase/AP-1 pathway is required for efficient apoptosis induced by vinblastine. *Cancer Res.* 2001; 61: 4450–8.
40. **Dong D, Ko B, Baumeister P, *et al.*** Vascular targeting and antiangiogenesis agents induce drug resistance effector GRP78 within the tumor microenvironment. *Cancer Res.* 2005; 65: 5785–91.
41. **Mhaidat NM, Thorne R, Zhang XD, *et al.*** Involvement of endoplasmic reticulum stress in Docetaxel-induced JNK-dependent apoptosis of human melanoma. *Apoptosis.* 2008; 13: 1505–12.
42. **Wang Y, Wang W, Wang S, *et al.*** Down-regulation of GRP78 is associated with the sensitivity of chemotherapy to VP-16 in small cell lung cancer NCI-H446 cells. *BMC Cancer.* 2008; 8: 372.
43. **Kim Y, Lillo AM, Steiniger SC, *et al.*** Targeting heat shock proteins on cancer cells: selection, characterization, and cell-penetrating properties of a peptidic GRP78 ligand. *Biochemistry.* 2006; 45: 9434–44.
44. **Li M, Wang J, Jing J, *et al.*** Synergistic promotion of breast cancer cells death by targeting molecular chaperone GRP78 and heat shock protein 70. *J Cell Mol Med.* 2008; Epub ahead of print. DOI: 10.1111/j.1582-4934.2008.00575.x
45. **Dong D, Ni M, Li J, *et al.*** Critical role of the stress chaperone GRP78/BiP in tumor proliferation, survival, and tumor angiogenesis in transgene-induced mammary tumor development. *Cancer Res.* 2008; 68: 498–505.
46. **Fu Y, Wey S, Wang M, *et al.*** Pten null prostate tumorigenesis and AKT activation are blocked by targeted knockout of ER chaperone GRP78/BiP in prostate epithelium. *Proc Natl Acad Sci USA.* 2008; 105: 19444–9.