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Preclinical report

Evaluation of GL331 in combination with paclitaxel: GL331's interference with paclitaxel-induced cell cycle perturbation and apoptosis

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Combination of selecting agents that act on different cellular mechanisms is a common strategy in cancer chemotherapy. GL331 is a new potent topoisomerase II (Topo II) poison; distinctly, paclitaxel is a microtubule-interfering cancer chemotherapeutic agent. In this study, we intended to evaluate the efficacy of combining GL331 with paclitaxel in cell killing and apoptotic induction in nasopharyngeal carcinoma NPC-TW01 cells. By MTT and internucleosomal DNA cleavage assays, we found that pretreatment or simultaneous treatment of NPC-TW01 cells with GL331 could significantly interfere with paclitaxel's cell killing and apoptosis-inducing activity. When the administration schedule was reversed, the cytotoxicity of GL331 was attenuated by paclitaxel pretreatment. The anti-cancer activity produced by combining GL331 with paclitaxel was obviously lower than the addition of the activities of two individual agents. NPC-TW01 cells were treated with GL331 and ^{3}H-labeled paclitaxel simultaneously or with GL331 before ^{3}H-labeled paclitaxel. In both conditions, GL331 did not reduce the [^{3}H]paclitaxel level in the cells, suggesting that GL331's interference with paclitaxel's cell-killing and apoptosis-inducing efficacy did not result from any inhibition of cellular uptake or retention of paclitaxel. In addition, we found that GL331-induced perturbation of cell cycle progression dramatically over-rode the patterns of mitotic arrest induced by paclitaxel, and the mechanism could be the inhibition of cyclin B1/CDC2 kinase and MAD2 checkprotein activities.

Key words: Apoptosis, cyclin B1/CDC2, G_2/M arrest, GL331, MAD2.

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Introduction

GL331 is a new semisynthetic compound derived from podophyllotoxin. Its chemical formula is 4′-demethyl-4β-(4″-nitroanilino)-4-desoxypodophyllotoxin with a molecular weight of 520.48.[1] GL331 shares many physico-chemical and biochemical properties with its homologous compound VP-16.[2] As with VP-16, GL331 exhibits less inhibitory activity against microtubule assembly, but instead is identified as a topoisomerase II (Topo II) poison. GL331 induces cancer cell death by causing Topo II-mediated DNA damage and, as opposed to VP-16, induces Ser-15-phosphorylated p53 in gastric carcinoma SC-M1 cells.[3] Phosphorylation of p53 on Ser-15 can be an important indicator in signaling DNA damage to the p53-dependent apoptosis pathway.[4] Possession of higher ability to induce phosphoSer-15-p53 seems to render GL331 more efficient to kill cancer cells via apoptosis.[3] In addition, GL331 has also shown higher efficacy to induce apoptosis in cancer cells harboring a p53 defect.[1,5,6] GL331-caused DNA strand breaks induce poly(ADP-ribose)polymerase (PARP) activation in HL-60 leukemia cells.[5] The nuclear enzyme PARP is responsible for the poly(ADP-ribosyl)ation of several apoptosis-related proteins.[7] In addition to acting as a Topo II poison, GL331 also induces activation of cellular CDC 25A phosphatase that results in transient cyclin B1/CDC2 activation and subsequent apoptosis in nasopharyngeal carcinoma NPC-TW01 cells.[8,9] The promising GL331 single-agent activity has promoted considerable interest in combining this drug with other anti-cancer agents.[10]

Paclitaxel is a potent cancer therapeutic agent for several malignancies, especially advanced breast, ovarian, and head and neck cancers.[11,12] By binding with tubulin molecules, paclitaxel enhances the

polymerization of tubulins, affecting the disassembly of spindle fibers and thus leading to cell cycle blockage in G_2/M.[13,15] However, the persistent G_2/M arrest and accompanying apoptosis occurs only in NPC-TW01 cells treated with higher concentrations of paclitaxel; at low doses or a short pulse, paclitaxel treatment renders NPC-TW01 cells in transient arrest at G_2/M before subsequent sub-G_1 apoptosis.[16,17] In considering persistent or transient G_2/M arrest is a prerequisite step for paclitaxel-induced NPC-TW01 cell apoptosis, some G_2/M events could be important and required for paclitaxel's anti-cancer activity. Previously, we found that cyclin B1/CDC2 activity was increased and persisted for more than 6 h in NPC-TW01 cells upon paclitaxel treatment both at high and low doses.[18] The maintenance of cyclin B1 for association with CDC2 is essential for the activation of CDC2 kinase. CDC 27-based anaphase-promoting complex (APC) is involved in the degradation of cyclin B1, but MAD2 inhibits this process by associating with APC.[19–22] Paclitaxel-induced MAD2/APC association accounts for the evasion of cyclin B1 degradation and thus the persistence of cyclin B1/CDC2 activation in the cells after paclitaxel treatment.[18]

Combination chemotherapy is a common strategy to treat cancer. It is considered for combination use if the selecting agents act on different cellular mechanisms.[23,24] Knowing that paclitaxel and GL331 have distinct anti-cancer mechanisms, we evaluated the efficacy of GL331's combination with paclitaxel in nasopharyngeal carcinoma NPC-TW01 cells. Our results indicated that GL331 and paclitaxel significantly interfered with each other in terms of cancer cell-killing and apoptosis-inducing activity. GL331 also prevented paclitaxel-induced patterns of mitotic arrest. The underlying mechanism could be the inhibition of cyclin B1/CDC2 kinase and MAD2 checkprotein activities.

Materials and methods

Cell line and reagents

Human nasopharyngeal carcinoma NPC-TW01 cells were cultivated in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.[25] GL331 and paclitaxel were obtained from Genelabs (Redwood City, CA) and Bristol-Myers Squibb (Syracuse, NY), respectively. The antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; anti-cyclin B1, CDC2, and MAD2) and Transduction Laboratories (Lexington, KY; anti-CDC 27).

MTT assay

Cytotoxicity of GL331 in combination with paclitaxel was evaluated by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.[9] NPC-TW01 cells (10^3) were seeded on each well of 96-well dishes and treated with various drug treatments in 200 μl of medium. After the treating periods, 50 μl of 0.25% MTT in PBS was added and incubated for another 4 h. Finally, media were removed and formazan was extracted with 50 μl/well of dimethyl sulfoxide. Quantitation was performed by a Spectra-Count reader (Packard, Meriden, CT) set at 550 nm wavelength.

Internucleosomal DNA cleavage assay

Apoptotic DNA fragments were isolated by the method described previously.[25] NPC-TW01 cells were treated as indicated for 24 h, and adherent and non-adherent cells were collected together for lysis by using 4°C pre-cold lysis buffer consisting of 50 mM Tris–HCl (pH 7.5), 20 mM EDTA and 1% NP-40. The cell lysates were then brought to 1% SDS and further treated for 1 h with 0.2 μg/μl RNase A at 45°C followed by overnight digestion with 2.5 μg/μl proteinase K at 37°C. After addition of ammonium acetate and glycogen, DNA was further extracted from cell lysates with phenol : chloroform (1 : 1) twice and chloroform once, and precipitated by −20°C pre-chilled ethanol. The DNA ladder patterns were obtained after 1% agarose gel electrophoresis and ethidium bromide staining, and the image was printed using an Alpha-InnoTech IS500 digital imaging system (Avery Dennison, Pasadena, CA).

Flow cytometric analysis of DNA content

Flow cytometry was performed as described previously.[17] NPC-TW01 cells treated as indicated were trypsinized and fixed with −20°C pre-chilled 80% ethanol. Cell pellets were then resuspended and incubated with 0.5% Triton X-100 for 5 min. The suspensions of permeabilized cells were further mixed with 1 ml of 50 μg/ml propidium iodide plus 0.5% (w/v) RNase A. Ten minutes later, the DNA content of cell samples was analyzed by a FACStar flow cytometer with an argon laser tuned to the 488 nm line for excitation (Becton Dickinson).

[^{3}H]Paclitaxel assay

The first set of cells was treated for 8 h with 2 μM of GL331 prior to a further 2-h treatment with 0.1 μM of

[^{3}H]paclitaxel (10 Ci/mmol; Moravek Biochemicals, Brea, CA). The second set of cells was treated for 2 h with 0.1 μM [^{3}H]paclitaxel and 0.2 or 2 μM of GL331 simultaneously. Both sets of cells were extensively washed with PBS, and then lyzed by the solution consisting of 0.5% SDS, 10 mM EDTA and 100 mM NaOH. Cellular [^{3}H]paclitaxel levels were quantitated by a liquid scintillation counter (Packard, Meriden, CT).

Immunoprecipitation, *in vitro* histone H1 kinase assay and Western blot analysis

Cell lysates were prepared by adopting a mild lysis method.[26] After the concentrations were determined by the Bradford method (BioRad, Hercules, CA), equal amounts of cell lysates were incubated with the indicated antibodies for immunoprecipitation at 4°C for 15 h. For *in vitro* histone H1 kinase assay, anti-cyclin B1 immunoprecipitates were washed 4 times with lysis buffer (50 mM Tris–Cl, pH 7.4, 250 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin A and 10 μg/ml leupeptin) and twice with kinase buffer (20 mM Tris–Cl, pH 7.4, 7.5 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 μg/ml bovine albumin serum).[8] The kinase reactions were performed in a vibrating shaker at room temperature for 30 min. After 10% SDS–polyacrylamide gel electrophoresis, proteins were electrotransferred onto PVDF membranes, and then the radioactive histone H1 bands were detected and printed by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). For Western blot analysis, the immunoprecipitates were washed 4 times with lysis buffer prior to typical gel electrophoresis and immunoblotting procedures.[1] The protein bands were finally detected by enhanced chemilluminescence (ECL; Amersham, Amersham–Pharmacia Biotech, Piscataway, NJ).

Results

Antagonism between GL331 and paclitaxel-induced cytotoxicities

The cytotoxicity of GL331's combination with paclitaxel in NPC-TW01 cells was evaluated by MTT assay. We observed that 2 μM of GL331 caused about 60% of NPC-TW01 cells to die after a 72-h incubation period (Figure 1A). This cytotoxicity was not enhanced by the presence of 1 nM–1 μM of paclitaxel; moreover, the efficacy of combined GL331 with paclitaxel was significantly lower than the addition of the activities of two individual agents (Figure 1A). If GL331 was added 8 h earlier than paclitaxel, the results were the same as those for GL331 added simultaneously with paclitaxel (Figure 1B). Conversely, when paclitaxel was added 8 h before GL331, the cytotoxicity curve was different (Figure 1C). However, after correcting for paclitaxel cytotoxicity, GL331 caused less cell killing in comparison with the control incubation without initial paclitaxel treatment. These data suggest that interference exists between GL331 and paclitaxel in killing NPC-TW01 cells.

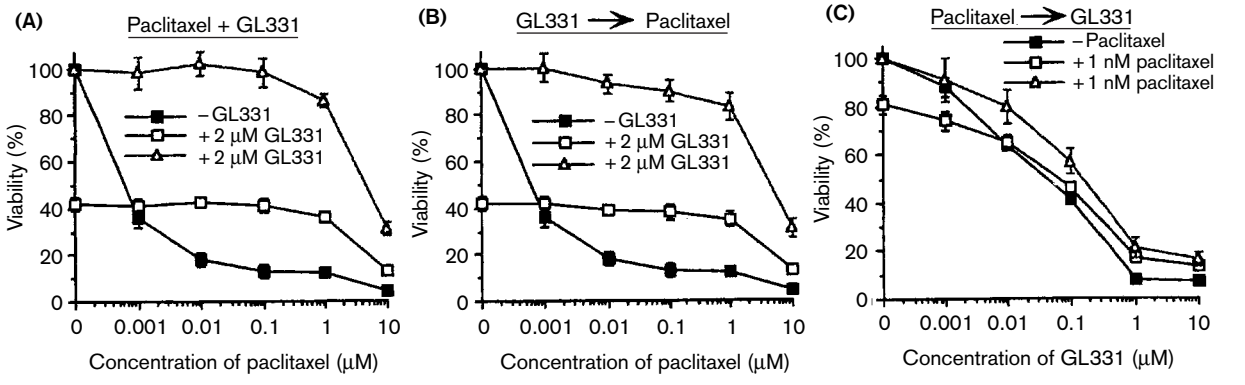

Figure 1. *Ex vivo* evaluation of the cytotoxicity of GL331 in combination with paclitaxel. (A) Survival curves of NPC-TW01 cells treated 72 h with varying doses of paclitaxel in the absence (closed squares) or presence (open square) of 2 μM of GL331. The open triangles represent viabilities corrected for GL331 cytotoxicity. (B) Survival curves of NPC-TW01 cells treated for 72 h with varying doses of paclitaxel in the absence (closed squares) or presence (open squares) of pretreatment with 2 μM of GL331 for 8 h. The open triangles represent viabilities corrected for GL331 cytotoxicity. (C) Survival curves of NPC-TW01 cells treated for 72 h with varying doses of GL331 in the absence (closed squares) or presence (open squares) of pretreatment with 1 nM of paclitaxel for 8 h. The open triangles represent viabilities corrected for paclitaxel cytotoxicity. All the data represent the means ± SD of three independent MTT assays. Each assay was performed in quintuple.

Antagonism between GL331 and paclitaxel-induced apoptosis

We furthermore investigated whether GL331 and paclitaxel would interfere with each other to induce apoptosis. In analyzing the DNA from NPC-TW01 cells treated with GL331 or paclitaxel, we found that 0.1 μM of paclitaxel is more efficient than 20 μM of GL331 to induce apoptotic DNA ladders (Figure 2). When GL331 and paclitaxel were added simultaneously, the level of 0.1 μM paclitaxel-induced DNA fragmentation was decreased by 0.2 and 2 μM of GL331. When the dose of GL331 was increased to 20 μM, the combination of GL331 with paclitaxel resulted in inhibition of any detectable DNA fragmentation levels induced by individual GL331 or paclitaxel alone (Figure 2), suggesting interference exists between GL331 and paclitaxel in inducing NPC-TW01 cell apoptosis.

GL331 interferes with paclitaxel-induced G_2/M arrest

Perturbation of cell cycle progression was associated with paclitaxel's anti-cancer mechanism. We found that paclitaxel-induced cell cycle perturbation, whatever the 0.1 μM-induced persistent G_2/M arrest or the transient G_2/M arrest caused by 5 nM, was interfered with by the presence of GL331 (Figure 3). The cell cycle distribution of the cells treated with paclitaxel plus GL331 was almost the same as those cells treated with GL331 alone. To exclude the possibility that GL331 would affect the cellular event(s) that in turn affect the cellular paclitaxel level, the effect of GL331 on cellular uptake or retention of paclitaxel was examined. NPC-TW01 cells were treated with GL331 and ^{3}H-labeled paclitaxel simultaneously, and the result shows that GL331 did not reduce the [^{3}H]paclitaxel level in the cells (Figure 4). Similar results were obtained if GL331 was added before paclitaxel (Figure 4), suggesting that the fact that GL331 over-rides paclitaxel to affect the cell cycle progression did not result from any inhibition of cellular uptake or retention of paclitaxel.

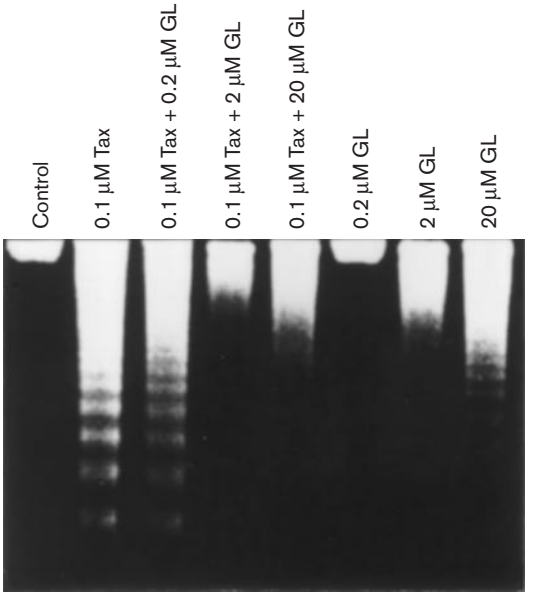

Figure 2. Agarose gel electrophoretic analysis of apoptotic DNA fragmentation from NPC-TW01 cells treated for 24 h with 0.1 μM paclitaxel (Tax) in the presence of 0.2, 2 or 20 μM GL331 (GL). Representative data from three independent experiments is shown.

GL331 interferes with paclitaxel-induced cyclin B1/CDC2 and MAD2 activation

We previously reported that GL331 induced a transient activation of cyclin B1/CDC2 kinase, which was immediately followed by disruption of the cyclin B1–CDC2 complex and thus inhibition of CDC2 kinase activity.[8,9] Therefore, we examined whether GL331 interfered with paclitaxel-induced persistent cyclin B1/CDC2 activation. The result shows that 0.1 μM paclitaxel-induced activation of cyclin B1/CDC2 was dramatically abolished in the presence of 2 or 20 μM GL331 (Figure 5A). A slightly increased cyclin B1/CDC2 activity was observed with the cells treated with 2 or 20 μM GL331 alone, which was probably the residual result of the transient activation that occurred during the initial 3-h incubation (data not shown). Moreover, paclitaxel-induced CDC27/MAD2 association was also effectively inhibited by GL331 (Figure 5B). The cellular MAD2 level was not affected by paclitaxel or GL331 treatment; the CDC27 level was increased by 0.1 μM paclitaxel and this elevation was obviously inhibited by GL331 (Figure 5B). As expected from the responses to CDC27/MAD2 association, the steady-state level of cyclin B1 was elevated in 0.1 μM paclitaxel-treated cells, but the elevation was prevented when GL331 was present in the treatment (Figure 5C).

Discussion

GL331 is a new congener of VP-16. The clinical therapeutic efficacy of VP-16 would be compromised once cancer cells expressed multidrug resistance, indicating a necessity to develop new effective therapeutic agents and schedules.[27] GL331 has

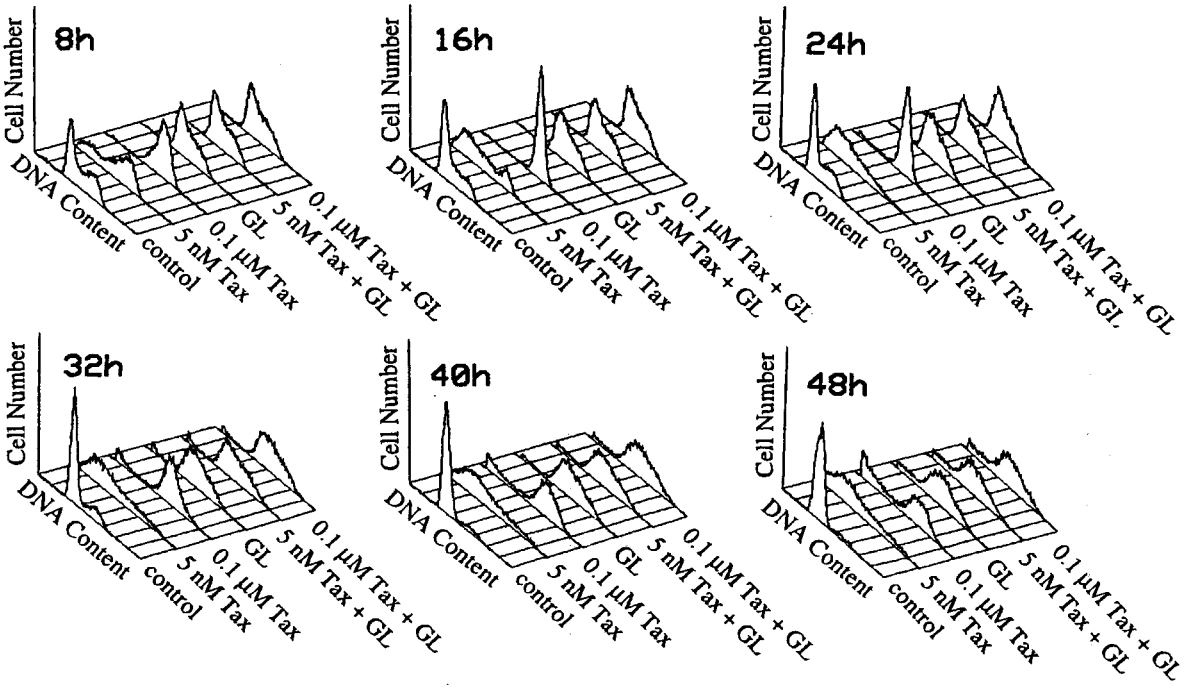

Figure 3. Flow cytometric analysis of DNA content from NPC-TW01 cells treated with 5 nM or 0.1 μM paclitaxel (Tax) in the absence or presence of 2 μM GL331 (GL) for different time points. Representative data from two independent experiments is shown.

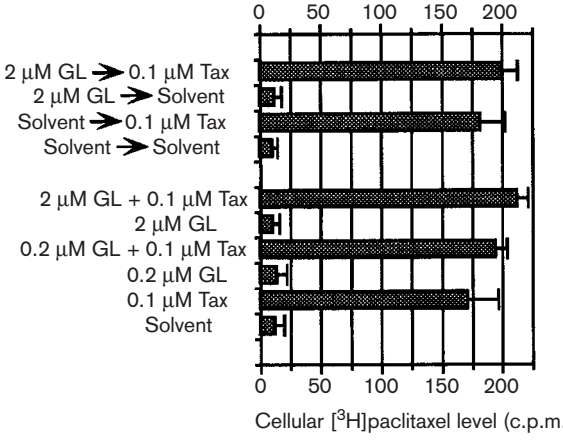

Figure 4. Measurement of cellular [^{3}H]paclitaxel levels from the NPC-TW01 cells treated simultaneously with GL331 and [^{3}H]paclitaxel for 2 h or treated for 8 h with GL331 before subsequent [^{3}H]paclitaxel for a further 2 h. Equal amounts of cell lysates were subjected to ^{3}H quantitation by liquid scintillation counting. The data represent the means $\pm$ SD of three independent assays. Each assay was performed in triplicate.

shown more efficacious anti-cancer activity both in *ex vivo* and *in vivo* drug-resistant lymphoma systems.[2] Its higher cytotoxicities and apoptosis-inducing activities were also demonstrated in several human cell lines from nasopharyngeal, hepatocellular, gastric, colon, cervical and neuroblastoma cancers that were not normally treated with VP-16.[6] The over-riding drug resistance and elevated ability to kill cancer cells suggest GL331's superiority in cancer therapy. In addition to demonstrating GL331's single-agent activity, in this study we used NPC-TW01 cells to evaluate the efficacy of GL331 in combination with paclitaxel. We know that when two drugs are combined, the outcome can be synergistic, additive or antagonistic. We found that pretreatment of NPC-TW01 cells with GL331 for 8 h significantly interfered with the cell-killing activity of paclitaxel. When the schedule of drug administration was reversed, 8-h pretreatment of paclitaxel also antagonized the cytotoxicity of GL331. In a separate study, we found that pretreatment of NPC-TW01 cells with GL331 could significantly antagonize the cytotoxicities caused by VP-16, 5-FU, adriamycin and cisplatin.[10] If these anti-cancer drugs were used before GL331, an enhancing or antagonistic effect was detected depending on the drug dose used. High-toxic doses of these drugs revealed an antagonistic effect on GL331; however, their low-toxic doses had an additive or synergistic effect on the cytotoxicity induced by GL331 at 0.1 μM or less; although for GL331 at greater than 1 μM, the effect

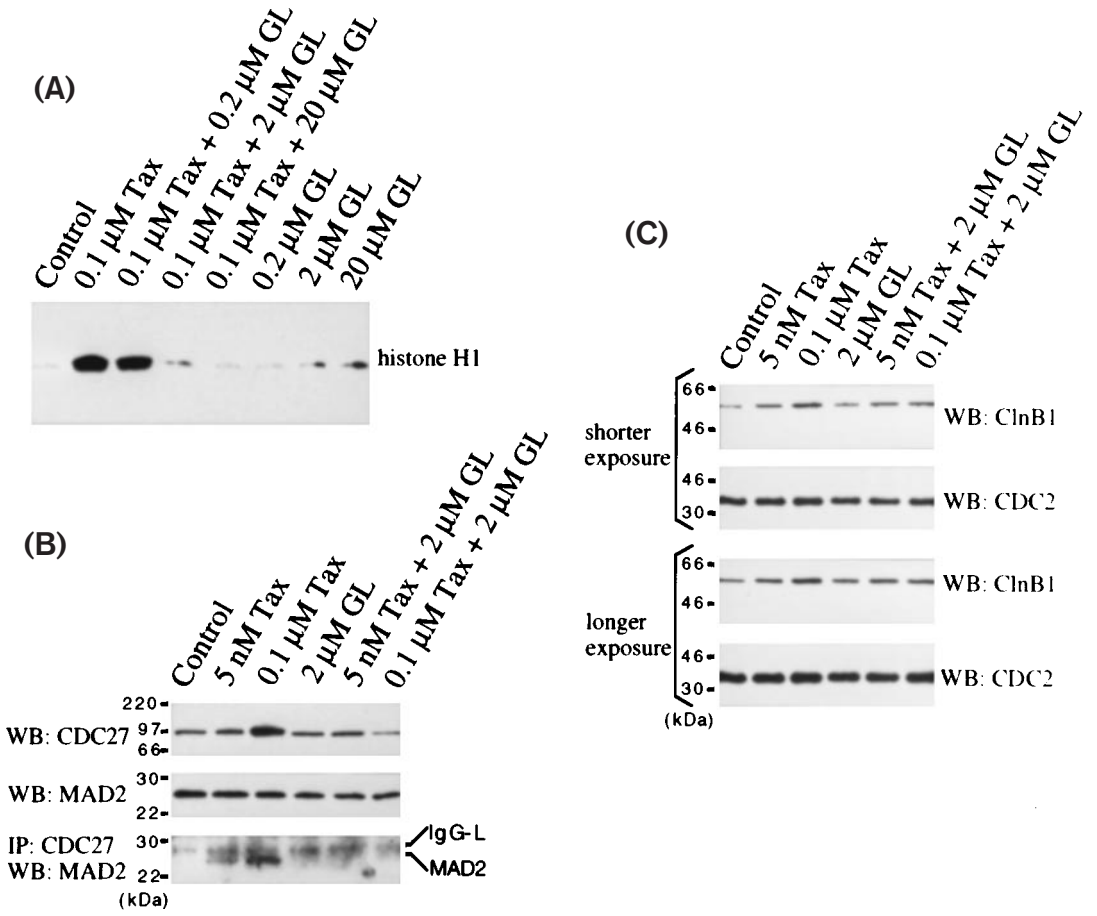

Figure 5. GL331 interferes with paclitaxel-induced activation of cyclin B1/CDC2 and MAD2. (A) Inhibition of *in vitro* histone H1 kinase activity of the cyclin B1/CDC2 from NPC-TW01 cells treated with 0.1 μM of paclitaxel in the presence of 0.2, 2 or 20 μM of GL331. (B) Inhibition of MAD2/CDC27 complex formation in NPC-TW01 cells treated with 5 nM or 0.1 μM of paclitaxel in the presence of 2 μM of GL331. The upper and middle panels show the results of anti-CDC27 and MAD2 Western blot analysis, respectively. The lower panel is the Western blot result of the MAD2 levels from anti-CDC27 immunoprecipitates. (C) Western blot analyses of the cyclin B1 and CDC2 levels in NPC-TW01 cells treated with paclitaxel and GL331. The upper two panels resulted from exposure within seconds and the lower two panels were obtained after being exposed for 1 min. All data are representative results from at least three independent experiments.

became antagonistic.[10] Although the promising anti-neoplastic efficacy of GL331 has promoted considerable interest in combining this agent with other therapeutic drugs, more preclinical studies will be necessary to determine the dose combination and administration schedules that will enhance rather than interfere with the individual anti-cancer activity.

Furthermore, the mechanism responsible for GL331's interference with paclitaxel cytotoxicity was investigated. It is considered that persistent or transient G_2/M arrest is a prerequisite step for paclitaxel-induced apoptosis,[16,17] and, clinically, VP-16 and 5-FU were shown to interfere with paclitaxel's cytotoxicity by blocking cellular entry into G_2/M arrest.[28,29] We observed that GL331 also interfered with paclitaxel-induced apoptosis by affecting its G_2/M-arrest induction and, moreover, the underlying mechanism could be GL331-induced prevention of MAD2 activity. Cyclin B1/CDC2 and MAD2 are two important molecules in regulating mitosis process. Cyclin B1/CDC2 is a critical kinase to control the progression of prophase and metaphase.[18] Inactivation of cyclin B1/CDC2 by APC is the only essential event required for mitotic exit.[30] APC is a CDC27-based ubiquitin ligase responsible for down-regulation of cyclin B1/CDC2 by the mediation of cyclin B1 degradation, and its activation has been shown to be required for metaphase–anaphase transition and for exit from mitosis. MAD2 is a spindle check protein that forms the complex with APC and thus prevents the APC's activity.[19–22] The spindle checkpoint mechanism is thought to guard the integrity of cell division by

monitoring interactions of the spindle fibers and the kinetochores. Once misaligned chromosomes are present, MAD2 inhibits the onset of anaphase until all chromosomes exhibit proper attachment to the spindle fibers.[19–22] The microtubule destabilizing drugs such as nocodazole also activate the MAD2-mediated spindle checkpoint. The MAD2 checkpoint appears to adopt a mechanochemical system that senses the presence or absence of physical tension mediated by microtubule attachment at the kinetochores of chromosomes. Through binding with tubulin molecules and further freezing microtubules, paclitaxel could induce the spindle checkpoint activity of MAD2 that accounts for evasion of cyclin B1 degradation and thus persistence of cyclin B1/CDC2 activation.[18]

In addition to MAD2 acting as a negative regulator, APC is activated by cell cycle-specific phosphorylation and complex formation with its positive regulators CDC20 and CDH1.[19–21] When cells enter mitosis, APC is phosphorylated under direct or indirect control of the cyclin B1/CDC2 kinase and phosphorylation increases the affinity for CDC20 to APC. The binding with CDC20 is essential for APC's further association with MAD2. Early in mitosis, MAD2, CDC20 and APC form a ternary complex, and this complex is inactive in ubiquitinating substrates. Anaphase is initiated by dissociation of MAD2 from the complex; APC is then activated by bound CDC20. Later in mitosis, CDH1 binds to and activates APC, which allows cells to exit from mitosis.[19–22] In our study, GL331 treatment inhibited paclitaxel-induced maintenance of MAD2/APC association probably via down-regulation of cyclin B1/CDC2 kinase, and thus prevention of the phosphorylation of APC and in turn the binding with CDC 20. A potent cyclin B1/CDC2 inhibitor flavopiridol could also efficiently inhibit MAD2/APC association (our unpublished data), supporting the probability that paclitaxel-induced cyclin B1/CDC2 activation was an essential step for paclitaxel-induced MAD2's binding and inhibition of APC. However, direct evidence interpreting the action of paclitaxel on the interactions among APC, CDC20 and MAD2 is still lacking.

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