Difference of cell cycle arrests induced by lidamycin in human breast cancer cells

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Lidamycin (LDM) is a member of the enediyne antibiotic family. It is undergoing phase I clinical trials in China as a potential chemotherapeutic agent. In the present study, we investigated the mechanism by which LDM induced cell cycle arrest in human breast cancer cells. The results showed that LDM induced G_1 arrest in p53 wild-type MCF-7 cells at low concentrations, and caused both G_1 and G_2/M arrests at higher concentrations. In contrast, LDM induced only G_2/M arrest in p53-mutant MCF-7/DOX cells. Western blotting analysis indicated that LDM-induced G_1 and G_2/M arrests in MCF-7 cells were associated with an increase of p53 and p21, and a decrease of phosphorylated retinoblastoma tumor suppressor protein, cyclin-dependent kinase (Cdk), Cdc2 and cyclin B1 protein levels. However, LDM-induced G_2/M arrest in MCF-7/DOX cells was correlated with the reduction of cyclin B1 expression. Further study indicated that the downregulation of cyclin B1 by LDM in MCF-7 cells was associated with decreasing cyclin B1 mRNA levels and promoting protein degradation, whereas it was only due to inducing cyclin B1 protein degradation in MCF-7/DOX cells. In addition, activation of checkpoint kinases Chk1 or Chk2 maybe contributed to LDM-induced cell cycle arrest. Taken together, we provide the first evidence that LDM induces different cell cycle arrests in human breast cancer cells, which are dependent on drug concentration and p53 status. These findings are helpful in understanding the molecular anti-cancer mechanisms of LDM and support its clinical trials. *Anti-Cancer Drugs* 17:173–179 © 2006 Lippincott Williams & Wilkins.

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Introduction

The cell cycle checkpoint refers to mechanisms by which the cell actively halts progression through the cell cycle until it can ensure that an earlier process, such as DNA replication or mitosis, is complete [1]. The tumor suppressor gene product p53 is one of the essential components of cell cycle checkpoints. p53 is a transcription factor that upregulates a number of important cell cycle-modulating genes, including cyclin-dependent kinase (Cdk) inhibitor p21. The ability of DNA-damaging agents to activate the G_1 checkpoint is caused at least in part by p53-dependent p21 activation [2–4]. Then, p21 suppresses cyclin E- and A-associated cdk2 activities, and thereby prevents G_1–S phase progression. p53 also regulates the G_2/M checkpoint by inducing the transcription of Gadd45, p21 and 14-3-3σ, and by repressing the transcription of cyclin B1 [5,6], which results in G_2/M cell cycle arrest.

In eukaryotes, cell cycle progression is regulated by sequential activation and subsequent inactivation of a series of Cdks. The activities of Cdks are positively regulated by cyclins, which act at different checkpoints of the cell cycle [7]. At G_1 phase, the cyclin D/Cdk4(Cdk6) complex is necessary for transition through early G_1, whereas the cyclin E/Cdk2 complex is required in transition from the G_1 to S phase [8–10]. Activated Cdks phosphorylate retinoblastoma tumor suppressor protein (pRb) in the G_1 phase, which results in the release of active E2F transcription factors to stimulate the transcription of genes involved in DNA synthesis and S-phase progression [11,12]. Cyclin B1 and Cdc2 (Cdk1) act at the G_2/M transition. In the G_2 phase, ATM (ataxia-telangiectasia-mutated) and ATR (ATM- and Rad-3-related protein kinase) activate the checkpoint kinases Chk2 and Chk1, respectively, which in turn inhibit Cdc25C by phosphorylating it on Ser216 [13,14]. In this case, Cdc25C cannot activate the Cdc2/cyclin B1 complex which induces G_2/M arrest [15].

Lidamycin (LDM, originally named C-1027), a member of the enediyne antibiotic family, was isolated from a *Streptomyces globisporus* C-1027 strain in China [16] and had extreme cytotoxicity toward human cancer cells [17–19]. It is currently being evaluated in phase I clinical trials as a potential chemotherapeutic agent. It has been

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reported that LDM can induce G_2/M cell cycle arrest in human cancer cells [19,20], but the detailed molecular mechanism of LDM-induced cell cycle arrest is still unclear. In the present study, we focused on the effect of LDM on cell cycle progression and aimed to elucidate its mechanism. We provide the first evidence that LDM induces different cell cycle arrests in MCF-7 and MCF-7/DOX cells, which are dependent on drug concentration and p53 status.

Methods

Cell culture and chemicals

Human breast cancer MCF-7 and MCF-7/DOX cells were cultured in DMEM (Gibco BRL, Grand Island, New York, USA) supplemented with 10% heat-inactivated FBS (Sigma, St Louis, Missouri, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. MCF-7/DOX cells were grown in medium containing 10 μmol/l doxorubicin (DOX) and passaged for 2 weeks in drug-free medium before each experiment. For synchronization, MCF-7 cells at a density of 60–70% were cultured in the serum-free medium for 48 h and released by adding 10% serum-repleted medium with or without LDM.

LDM was generously provided by Professor Jin (Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Beijing, China). LDM stock solution (10 μmol/l) was prepared in water and stored at −70°C. LLnL (calpain inhibitor I), propidium iodide (PI) and RNase A were purchased from Sigma. All other chemicals were of standard analytical grade.

Antibodies

Mouse monoclonal anti-cyclin B1 (GNS1), rabbit polyclonal anti-Cdc2 (C-19), mouse monoclonal anti-p21 (F-5), mouse monoclonal anti-cyclin D1(A-12), rabbit polyclonal anti-Cdk2 (M2) and goat polyclonal anti-actin (I-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Mouse monoclonal anti-p53 (2524), rabbit polyclonal anti-phospho-pRb(Ser807/811), rabbit polyclonal anti-chk1(2345), rabbit polyclonal anti-chk2 (2662), rabbit polyclonal anti-phospho-chk1 (Ser345) (2341) and rabbit polyclonal anti-phospho-chk2 (Thr68) (2661) antibodies were from Cell Signaling Technology (Beverly, Massachusetts, USA).

Cell cycle analysis

Cells were collected, fixed in ice-cold 70% ethanol and stored at −20°C prior to analysis. Samples were washed twice in PBS, and resuspended in a solution of PI (50 μg/ml) and RNase A (0.5 mg/ml) in PBS for 30 min in the dark. The stained cells were filtered through 40-μm gauze and the single-cell suspensions were analyzed on an Epics XL flow cytometer (Coulter, Miami, Florida, USA) using Mcycle software.

Western blotting

Cells were harvested, washed with PBS solution, and lysed with buffer containing 50 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 2 mmol/l EDTA, 2 mmol/l EGTA, 1 mmol/l dithiothreitol, 1% Nonidet P-40, 0.1% SDS, protease inhibitors (1 mmol/l PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml pepstatin) and phosphatase inhibitors (20 mmol/l β-glycerophosphate, 50 mmol/l NaF and 1 mmol/l Na_3VO_4). Lysates were incubated for 20 min at 4°C and centrifuged at 12 000 g for 12 min. Protein concentrations were determined by the Bradford assay. Equal amounts of lysate (40 μg) were resolved by SDS–PAGE and transferred to PVDF membranes (Millipore, Bedford, Massachusetts, USA). Membranes were blocked in 5% non-fat skim milk/TBST [20 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl and 0.1% Tween-20] at room temperature for 2 h and probed with primary antibodies overnight at 4°C. Then membranes were blotted for 1 h at room temperature with an appropriate horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology). Proteins were visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

RT-PCR

Total RNA was extracted using a SuperScript One-Step RT-PCR kit (Invitrogen, Carlsbad, California, USA) as described by the manufacturer. The following primers were used for PCR: B1-hum-for, 5′-AAGAGC TTTAAAC TTTGGTCTGGG-3′; B1-hum-rev, 5′-CTTTG TAAGT CCTTGATTTACCATG-3′ [6]; β-actin P1, 5′-CCCAGG-CACCAGGGCGTGATGGT-3′; β-actin P2, 5′-GGACTC-CATGCCCAGGAAGGAA-3′. Reverse transcription was performed at 55°C for 15 min. The denaturation and amplification conditions were 95°C for 30 s followed by up to 25 cycles of PCR. Each cycle of PCR included denaturation at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 40 s. After PCR amplification, the fragments were analyzed by 2% agarose gel electrophoresis.

Results

Biphasic response of MCF-7 cells to LDM is dependent on drug concentration

First, we investigated the effect of LDM on cell cycle progression. Drug concentrations used were based on the published data [19]. Asynchronous MCF-7 cells were treated with different concentrations of LDM (0.01–1 nmol/l) for 24 h and then cell cycle distribution was determined. The results indicated that MCF-7 cells exhibited a biphasic response to LDM (Table 1). When treatment with 0.01 nmol/l LDM, the G_1 population was increased from 60.7 to 79.8%, indicating that LDM induced G_1 arrest. However, the G_2/M population was increased when the concentration was increased to 0.1 and 1 nmol/l (29.6 and 32.8%, respectively) compared

with untreated cells (9.8%), and this increase was only accompanied by a decrease of S-phase cells without any obvious reduction of the G_1 population (Table 1), indicating LDM might induce both G_1 and G_2/M arrests at these concentrations. Similar results were also observed when exposure to different concentrations of LDM for 48 h (data not shown).

To further confirm that LDM could induce G_1 arrest in MCF-7 cells at higher concentrations (0.1 and 1 nmol/l), cells were synchronized by serum starvation for 48 h and then released. Serum deprivation of MCF-7 cells for 48 h induced a significant increase of cells in the G_1 phase (83.5%, Fig. 1), indicating that most of the cells were synchronized at the G_1 phase. After 24 h release by serum repletion, control cells (without LDM) exhibited a significant reduction in the G_1 population (36.5%). Under the same conditions, most of cells treated with 0.1 nmol/l LDM still accumulated in the G_1 phase (82.3%).The results were similar when cells were treated with 1 nmol/l LDM (data not shown). These data verify that LDM can induce G_1 arrest at higher concentrations in MCF-7 cells.

Table 1 Cell cycle distribution of MCF-7 cells after treatment with LDM

Concentration (nmol/l)	Cell cycle distribution (%)		
	G_1	S	G_2/M
0	60.7 ± 4.5	29.3 ± 3.8	9.8 ± 1.6
0.01	79.8 ± 3.7	14.5 ± 2.6	5.7 ± 1.3
0.1	58.5 ± 5.4	11.9 ± 2.4	29.6 ± 2.6
1	55.6 ± 3.3	11.6 ± 1.7	32.8 ± 4.3

MCF-7 cells were treated with LDM for 24 h and the cell cycle distribution was then examined as described in Materials and methods. Results are means ± SD of three independent experiments.

LDM induces only G_2/M arrest in MCF-7/DOX cells

MCF-7/DOX (also named MCF-7/ADR) cells were derived from MCF-7 cells by culturing them in the continuous presence of increasing doxorubicin concentrations [21]. However, unlike parental MCF-7 cells, MCF-7/DOX cells are multidrug resistant and p53-mutant [21–23]. Since p53 is one of the main regulators of the cell cycle checkpoint, we investigated whether this mutation would influence the response of cells to LDM. We found that LDM caused MCF-7/DOX cells to accumulate in the G_2/M phase in a concentration-dependent manner (Table 2). When exposed to 1 nmol/l LDM for 24 h, the cells in the G_2/M phase increased from 15.5 to 71.8% with a little G_1 population (5.5%). These data indicate that LDM induces only G_2/M arrest in p53-mutant MCF-7/DOX cells.

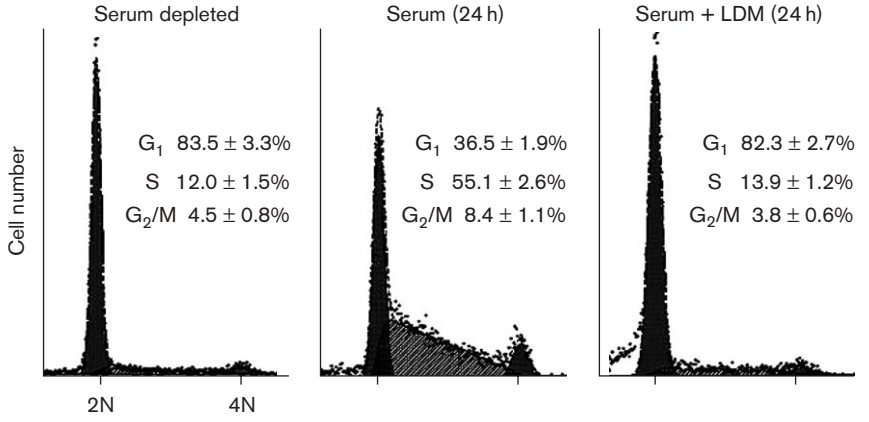
LDM increases p53 and p21 expression in MCF-7 cells

A common mechanism of cell cycle arrest in the G_1 phase involves activation of p53 and upregulation of p21. In addition, p53 also contributes to G_2/M arrest. To prove that LDM-induced dual cell cycle arrests in MCF-7 cells were associated with p53 activation, we next investigated p53 and p21 expression in these two cell lines. Western blotting results indicated that p53 and p21 protein levels increased dramatically after exposure to LDM for 24 h in MCF-7 cells (Fig. 2a). In contrast, p53 and p21 levels were unchanged in MCF-7/DOX cells in which LDM induced only G_2/M arrest (Fig. 2a).

Changes of cell cycle regulators after LDM treatment

To further prove that LDM induced simultaneous G_1 and G_2/M arrests in MCF-7 cells, we then investigated the changes of cell cycle regulators responsible for the G_1 and G_2/M phase, respectively. The protein levels of cyclin D1, Cdk2 and phosphorylated pRb (inactivated protein,

Fig. 1



LDM induces G_1 arrest in synchronized MCF-7 cells. Cells were cultured in serum-free medium for 48 h (serum depleted) and then released by adding 10% fresh serum medium with (serum + LDM) or without (serum) LDM for 24 h. Cell cycle distribution was then determined by flow cytometry. Data are representative of three independent experiments.

Table 2 Cell cycle distribution of MCF-7/DOX cells after treatment with LDM

Concentration (nmol/l)	Cell cycle distribution (%)		
	G_1	S	G_2/M
0	55.5 ± 4.2	29.0 ± 3.6	15.5 ± 4.5
0.01	52.1 ± 5.3	28.6 ± 4.2	19.3 ± 3.7
0.1	20.5 ± 3.8	26.0 ± 3.4	53.5 ± 4.6
1	5.5 ± 0.8	22.7 ± 2.5	71.8 ± 3.1

MCF-7/DOX cells were treated with LDM for 24 h and the cell cycle distribution was then examined as described in Materials and methods. Results are means ± SD of three independent experiments.

phosphorylation at Ser807/811) which regulated G_1 cell cycle checkpoint were examined by Western blotting. The results showed that the levels of cyclin D1 were increased at 0.01 nmol/l, while they were unchanged at 0.1 and 1 nmol/l compared with untreated cells (Fig. 2b). Cdk2 and phosphorylated pRb were both decreased after LDM treatment (Fig. 2b). These data are consistent with G_1 arrest.

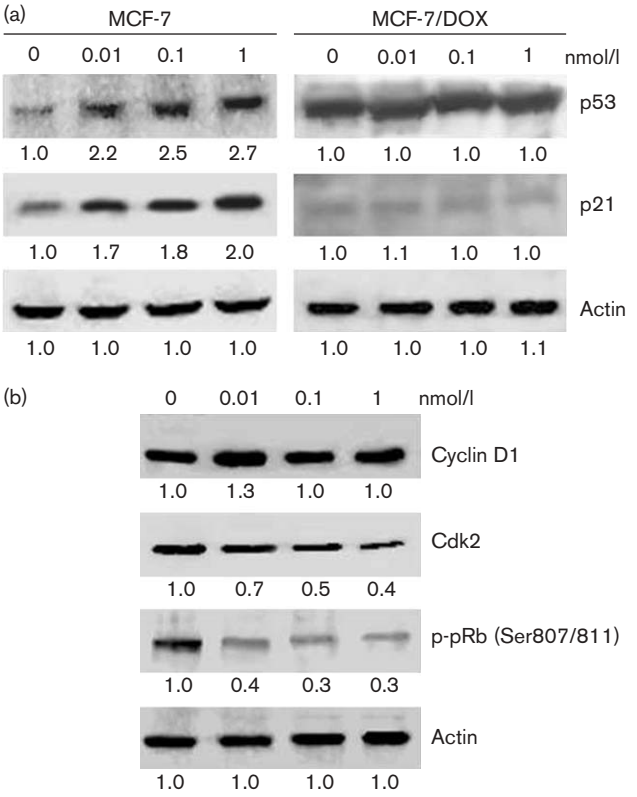
To prove that LDM induced G_2/M arrest in MCF-7 cells, we examined the expression of Cdc2 and cyclin B1, which are positive regulators for G_2/M transition [24,25]. After exposure to LDM for 24 h, the protein levels of cyclin B1 and Cdc2 were both decreased (Fig. 3a). In particular, cyclin B1 and Cdc2 expression was decreased dramatically at 0.1 and 1 nmol/l, which was consistent with G_2/M arrest. In addition, we also investigated the changes of cyclin B1 and Cdc2 in MCF-7/DOX cells. Similar to the results observed in MCF-7 cells, the protein levels of cyclin B1 were decreased dramatically after LDM treatment in MCF-7/DOX cells, whereas Cdc2 was slightly increased after exposure to 0.01 and 0.1 nmol/l LDM or unchanged at 1 nmol/l (Fig. 3a).

LDM downregulates cyclin B1

Based on the above results, we further investigated the mechanism by which LDM decreased the protein levels of cyclin B1. Using a semiquantitative RT-PCR assay, we first examined cyclin B1 mRNA levels. We found that cyclin B1 mRNA levels were decreased in MCF-7 cells when treated with 0.1 nmol/l LDM for 24 h, whereas cyclin B1 mRNA levels were unchanged in MCF-7/DOX cells under the same conditions (Fig. 3b).

What causes the reduction of cyclin B1 protein in MCF-7/DOX cells? It is recognized that proteolysis plays a critical role in the regulation of cell cycle progression [26] and cyclin B1 is one of the first proteins to be identified as a substrate for ubiquitin-mediated proteolysis [27]. According to these reports, we suppose that LDM maybe decreases the protein levels of cyclin B1 by promoting protein degradation in MCF-7/DOX cells. After cotreatment with LDM and LnLL, a calpain inhibitor that also blocks activity of the 26 S proteasome [28], the reduction

Fig. 2



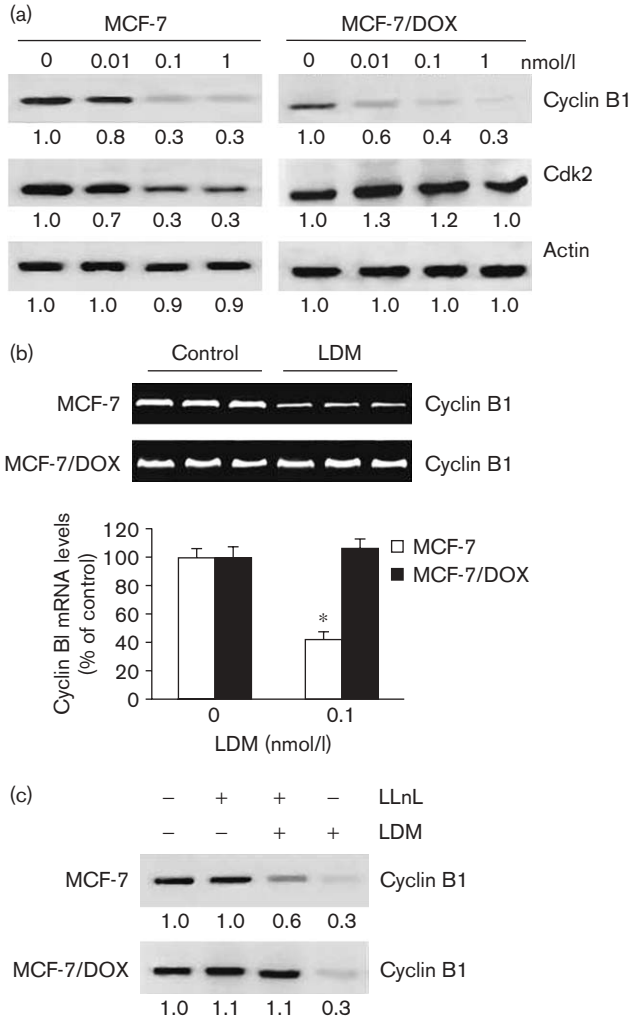
Expression of p53, p21 and G_1 cell cycle regulators. (a) MCF-7 (left panel) or MCF-7/DOX (right panel) cells were treated with LDM at the indicated doses for 24 h, and p53 and p21 protein levels were then determined by Western blotting as described in Materials and methods. Fold induction of protein level was based on densitometric measurements and is shown below each immunoreactive band. Actin was used as a standard for each sample and protein levels in untreated cells were defined as 1.0. Data are representative of three independent experiments. (b) MCF-7 cells were treated with LDM at the indicated doses for 24 h, and cyclin D1, Cdk2 and phospho-pRb (p-pRb) protein were then determined by Western blotting. Data are representative of three independent experiments.

of cyclin B1 expression induced by LDM was completely reversed in MCF-7/DOX cells compared with untreated cells (Fig. 3c). Unexpectedly, the combination of LnLL with LDM also partially increased cyclin B1 levels compared with LDM treatment alone in MCF-7 cells (Fig. 3c). These data suggest that the downregulation of cyclin B1 protein by LDM is associated with decreasing cyclin B1 mRNA levels and promoting protein degradation simultaneously in MCF-7 cells, whereas it is only due to inducing degradation of cyclin B1 protein in MCF-7/DOX cells.

LDM induces phosphorylation of Chk1 and Chk2

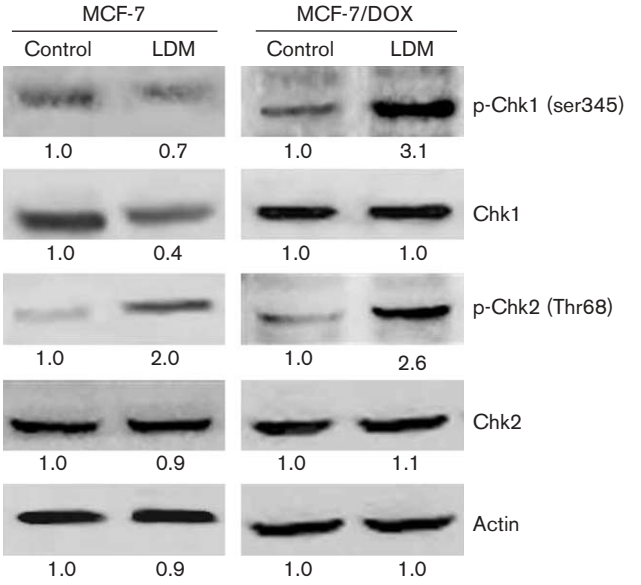
ATM phosphorylates Chk2 on Thr68 leading to Chk2 kinase activation [29], while both ATM and ATR phosphorylate Chk1 on Ser317 and Ser345, resulting in its activation [30,31]. Activated Chk1 and Chk2 phosphorylate Cdc25C on Ser216, and inhibit the ability

Fig. 3



Downregulation of cyclin B1 by LDM. (a) Expression of cyclin B1 and Cdc2. Cells were treated as described in the legend to Fig. 2, and then cyclin B1 and Cdc2 levels were determined by Western blotting. Data are representative of three independent experiments. (b) RT-PCR analysis of cyclin B1 mRNA levels. Cells were untreated or treated with 0.1 nmol/l LDM for 24 h and cyclin B1 mRNA levels were then determined as described in Materials and methods. The upper panel shows a representative of three independent experiments, with each experiment performed in triplicate. β-Actin served as internal loading controls. Each bar represents the mean ± SD cyclin B1 mRNA levels of three independent experiments and the mean cyclin B1 mRNA level of control was designated as 100% in the graph. $^{*}P \leq 0.01$ compared with control. (c) LDM promotes cyclin B1 protein degradation. Cells were untreated (−) or treated (+) with 0.1 nmol/l LDM or cotreated with LDM and 100 μmol/l LLnL for 24 h, and cyclin B1 expression was then evaluated by Western blotting. Data are representative of three independent experiments

Fig. 4



LDM induces phosphorylation of Chk1 and Chk2. MCF-7 (left panel) or MCF-7/DOX (right panel) cells were untreated (control) or treated with 0.1 nmol/l LDM (LDM) for 24 h, and the levels of Chk1, Chk2, phosphorylated Chk1 and phosphorylated Chk2 were then determined by Western blotting. Data are representative of three independent experiments.

of Cdc25C to activate Cdc2/cyclin B1, resulting in G_2/M cell cycle arrest [15]. Thus, we investigated whether LDM-induced G_2/M arrest involved the activation of these two pathways. In the present study, we only examined the changes of Chk1 and Chk2, but our future works will focus on other regulators. The results showed that phosphorylated Chk1 (Ser345) was decreased in MCF-7 cells, whereas it was increased in MCF-7/DOX cells after treatment with 0.1 nmol/l LDM for 24 h. Although Chk2 was unchanged, phosphorylated Chk2 (Thr68) was increased dramatically in both cell lines after exposure to 0.1 nmol/l LDM for 24 h (Fig. 4).

Discussion

To the best of our knowledge, this is the first attempt to elucidate the mechanism of LDM-induced cell cycle arrest in human cancer cells. Our results indicated that LDM induced different cell cycle arrests in MCF-7 and MCF-7/DOX cells. At low concentration (0.01 nmol/l), LDM induced only G_1 arrest; at higher concentrations (0.1 and 1 nmol/l), LDM caused both G_1 and G_2/M arrests (Table 1) in MCF-7 cells. These data suggest that the response of MCF-7 cells to LDM is dependent on drug concentration. With increasing concentration, LDM induces G_1 arrest (0.01 nmol/l), G_1 and G_2/M arrests (0.1 and 1 nmol/l) or apoptosis (above 1 nmol/l, data not shown), respectively. Because we were only interested in the cell cycle in the present study, we do not show any data for apoptosis. The following investigation indicated that LDM induced only G_2/M arrest in MCF-7/DOX cells (Table 2). Considering that MCF-7/DOX is a p53-mutant cell line, we presume that different cell cycle arrests induced by LDM in these two cell lines are correlated with the different p53 status.

To further confirm that p53 influenced the response of cells to LDM, we examined the p53 and p21 protein levels by Western blotting. As expected, MCF-7/DOX cells constitutively overexpressed p53 and there was no apparent change after LDM treatment (Fig. 2a), which was consistent with the previous studies that mutant forms of p53 were generally more stable and showed weak or no response to DNA damage in cancer cells [22,32,33]. The level of p21 was also unchanged after LDM treatment in MCF-7/DOX cells. In addition, we compared p53 expression of MCF-7/DOX cells growing in the presence of 10 μmol/l doxorubicin for 2 weeks with the same cells passaged for 2 weeks in doxorubicin-free medium. We found that both of them overexpressed p53, indicating p53 was mutated and the p53 status was unchanged after doxorubicin was removed from the medium (data not shown). In contrast, p53 and p21 levels were increased dramatically after exposure to LDM in p53 wild-type MCF-7 cells. At 0.01 nmol/l, which was the concentration that only induced G_1 without G_2/M arrest (Table 1), p53 and p21 were already increased by LDM (Fig. 2a). These data suggest that p53 and p21 may be more important in regulating LDM-induced G_1 arrest. After treatment with LDM, p53 is activated and then induces p21 expression, which results in G_1 arrest in MCF-7 cells. Since LDM induced G_2/M arrest without changing the p53 and p21 expression in p53-mutant MCF-7/DOX cells, we concluded that LDM could induce G_2/M arrest via a p53-independent pathway.

To prove LDM induced dual cell cycle arrests at G_1 and G_2/M phase in MCF-7 cells at higher concentrations, we examined the cell cycle regulators of G_1/S and G_2/M checkpoints, respectively. The Rb family of proteins cooperate to regulate cell cycle progression through the G_1 phase of the cell cycle [11]. Each of the Rb family proteins is negatively regulated by phosphorylation by Cdks, which frees the E2F family of transcription factors to induce the transcription of genes whose protein products are necessary for S-phase progression. Phosphorylation of pRb in mid-G_1 is thought to be due to cyclin D/Cdk4 and cyclin E/Cdk2 [34,35]. Consistent with these notions, the levels of phosphorylated pRb and Cdk2 were decreased after LDM treatment, providing the supporting data for the G_1 arrest found in MCF-7 cells. The reduction of cyclin B1 and Cdc2 was consistent with the G_2/M arrest [36,37]. It should be noted that cyclin B1 and Cdc2 were decreased at 0.01 nmol/l, which may be due to most of the cells accumulating in the G_1 phase [38]. When the concentration increased to 0.1 and 1 nmol/l, cyclin B1 and Cdc2 were decreased dramatically (Fig. 3a), which resulted in G_2/M arrest.

In addition, the protein levels of cyclin B1 were also reduced dramatically in MCF-7/DOX cells in which LDM induced only G_2/M arrest. Since the expression of cyclin B1 is one of the regulatory components of cyclin B1/Cdc2 activity [10], perhaps these results suggest that downregulation of cyclin B1 is a main step in the LDM-induced G_2/M arrest in MCF-7/DOX cells. It has been reported that p53 can repress transcription of cyclin B1 [6]. This might explain why cyclin B1 mRNA levels were decreased in MCF-7 cells (p53 wild-type), whereas they were unchanged in MCF-7/DOX cells (p53-mutant) after exposure to LDM (Fig. 3b). Furthermore, our results indicated that promoting cyclin B1 protein degradation was also a mechanism by which LDM downregulated cyclin B1.

Most DNA-damaging agents induce G_2/M cell cycle arrest through ATR/ATM–Chk1/Chk2–Cdc25C–Cdc2 checkpoint pathways [15,31,39]. The ATM/Chk2 pathway is principally activated by double-stranded DNA breaks, whereas the ATR/Chk1 pathway primarily responds to lesions caused by UV and DNA replication block [15,31,40]. Previous studies have reported that LDM induced high levels of double-strand breaks and inhibited DNA replication [41–43], implying that LDM might activate both of these pathways. Western blotting analysis showed that Chk1 and phosphorylated Chk1 were decreased, whereas phosphorylated Chk2 was increased dramatically in MCF-7 cells after exposure to LDM (Fig. 4), indicating that LDM activated Chk2 in MCF-7 cells. A previous study reported that p53 downregulated Chk1 through p21 [44], which might explain why LDM decreased the levels of Chk1 and phosphorylated Chk1 in MCF-7 cells. However, phosphorylated Chk1 and phosphorylated Chk2 were both increased in p53-mutant MCF-7/DOX cells in response to LDM, indicating both of them were activated by LDM. These data suggest that activation of Chk1 or Chk2 may contribute to LDM-induced G_2/M arrest. Our future work will continue to investigate these pathways.

Taken together, our study elucidates for the first time the mechanism of LDM-induced cell cycle arrest. These findings have important implications for understanding the molecular anti-cancer mechanism of LDM and provide more evidence to support using LDM as a chemotherapeutic agent in clinical cancer therapy.

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