Reversal effect of BM-cyclin 1 on multidrug resistance in C-A120 cells

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In this study, multidrug-resistant human epidermoid C-A120 cells and the sensitive parental KB cells were used as experimental models. BM-cyclin 1, a traditional antimycoplasma drug, was tested to explore the reversal effect of multidrug resistance and its mechanisms in these cell lines. The MTT analysis showed that BM-cyclin 1 could reverse multidrug resistance effectively in C-A120 cells; the sensitivity of C-A120 cells to adriamycin, etoposide and cisplatin was enhanced by 6.0, 8.2 and 1.7 times, respectively. Immunoblotting analysis and reverse transcription-polymerase chain reaction were used to study the BM-cyclin 1-induced changes in topoisomerase IIα. The results showed that the expression of topoisomerase IIα in treated C-A120 cells increased significantly. Topoisomerase II catalytic activity increased by 30% compared with the untreated cells, as measured by decatenation of kinetopolast DNA. Immunoblotting analysis also indicated the transcription factor levels of specificity: those of protein 1 (Sp1) and nuclear factor-YA increased after treatment with BM-cyclin 1, whereas the mRNA and protein expression of multidrug resistance protein 2 was significantly downregulated. These results demonstrated that BM-cyclin 1 could effectively reverse the multidrug resistance of C-A120 cells by increasing the expression of topoisomerase IIα and by suppressing the expression of multidrug resistance protein 2, strongly suggesting that BM-cyclin 1 is a potential multidrug resistance reversal agent. ***Anti-Cancer Drugs*** **18:1015–1021 © 2007 Lippincott Williams & Wilkins.**

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Introduction

Overexpression of P-glycoprotein (P-gp) or multidrug resistance (MDR) proteins (MRPs) has been demonstrated to be and implicated in mechanisms of resistance in some cancer-cell lines. P-gp and MRP_S belong to the ATP-binding cassette superfamily of transport proteins. These proteins induce MDR either by decreasing the total intracellular retention of drugs or by redistributing intracellular accumulation of drugs away from the target organelles. Another atypical MDR phenotype has been identified; the predominant mechanism of resistance of this phenotype has been shown to bring about qualitative or quantitative changes in the levels and activity of topoisomerase II (Topo II) [1]. The decreased expression of Topo IIα has been observed in a variety of cell lines that have developed resistance to a wide range of chemotherapeutic drugs. Mechanisms resulting in the downregulation of Topo IIα include transcriptional downregulation [2]. Recent studies showed that Topo IIα promoter activity is lower in the resistant cell lines [3].

C-A120 cell lines are non-P-gp-mediated resistant human KB cells. They were resistant to adriamycin (ADM), vincristine, cisplatin and etoposide (VP-16) [4]. Many studies indicated that both the increased expression of MRP1 and MRP2, and the decreased expression of Topo IIα underlie the drug resistance in C-A120 cells [4–7]. Although P-gp-mediated MDR can be reversed by a variety of compounds, most of these are not effective as agents reversing non-P-gp-mediated MDR [8]. Therefore, it is necessary to identify agents that could reverse the non-P-gp-mediated MDR. We found that BM-cyclin 1 can effectively reverse the resistance in C-A120 cells. BM-cyclin 1, a pleuromutilin derivative, has been reported to be the treatment of choice for the elimination of mycoplasmas from contaminated hybridomas [9] and other cell lines.

In our study, cell-growth inhibition assays showed that BM-cyclin 1 could increase the cell-killing activity of ADM, VP-16 and cisplatin in C-A120 cells. The results also demonstrated that BM-cyclin 1 could increase the expression of Topo IIα and suppress the expression of MRP2. The catalytic activity of Topo IIα and the effect of BM-cyclin 1 on the transcription factors of Topo IIα were also tested.

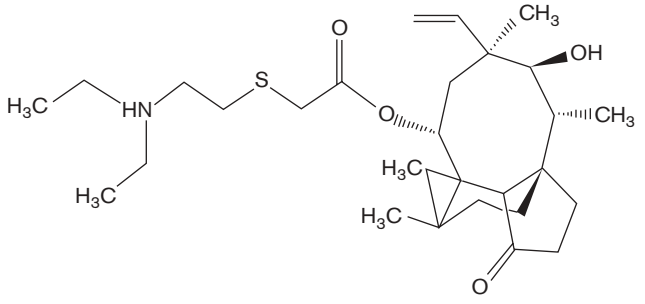
Materials and methods

Chemicals

BM-cyclin 1 was purchased from Roche (Nonnenwald, Penzberg, Germany). The chemical structure of

0959-4973

Fig. 1



BM-cyclin 1.

BM-cyclin 1 is shown in Fig. 1. The following primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA): Topo IIα, Sp1, Sp3, MRP2 and nuclear factor-YA (NF-YA); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Sigma-Aldrich (St Louis, Missouri, USA), kinetoplast DNA (kDNA) from Topogen (Columbus, Ohio, USA) and cell-culture reagents from Invitrogen (Carlsbad, California, USA).

Cells and cultures

Human epidermoid KB carcinoma cells and the KB cells were cultured in RPMI-1640 medium with penicillin (100 µl/ml), supplemented with 10% fetal calf serum at 37°C in 5% CO_2. KB cells were the drug-sensitive parental cell line. Non-P-gp-mediated resistant C-A120 cells were derived from human epidermoid KB carcinoma cells. The C-A120 cells were maintained in the medium with 120 ng/ml ADM, as described [4].

Cell survival by the MTT assay

Chemosensitivity *in vitro* was measured by means of the MTT colorimetric assay performed in 96-well plates. Equal numbers of cells (5000 for KB cells, 10 000 for C-A120 cells) were inoculated into each well with 80 µl of culture medium. After an overnight incubation (37°C with 5% CO_2), various concentrations of 20 µl of VP-16, ADM and cisplatin solution, with or without reversing agent, was added to the cultures and incubated for 4 days. Thereafter, 50 µl of MTT (1 mg/ml phosphate-buffered saline) was added to each well and incubated for an additional 4 h. The resulting formazan was dissolved with 100 µl of dimethylsulfoxide after aspiration of the culture medium. Plates were placed on a plate shaker for 5 min and read immediately at 570 and 630 nm using an enzyme-linked immunosorbent assay reader (Thermo Labsystems, Franklin, Massachusetts, USA). Experiments were performed in triplicate. From these results, the percentage of live cells in each well could be estimated and plotted against the drug concentrations as dose–response curves from which the IC_{50} was derived.

Kinetoplast DNA decatenation assays

Nuclear extracts were prepared from cells as described previously [10]. Following trypsinization, cells were washed in ice-cold phosphate-buffered saline–ethylene-diaminetetraacetic acid (EDTA), resuspended in 0.25 ml lysis buffer (20 mmol/l Tris–HCl, pH 7.2; 25 mmol/l KCl; 5 mmol/l MgCl2; 1 mmol/l of ethylene glycol-bis (b-aminoethyl ether); 250 mmol/l of sucrose and 0.5% Nonidet P-40) and kept on ice for 10 min. After a 2-min centrifugation in a microfuge, nuclei were resuspended in 0.25 ml nuclei buffer (20 mmol/l of Tris–HCl, pH 7.2; 400 mmol/l of NaCl; 20 mmol/l of EDTA and 20 mmol/l of 2-mercaptoethanol) and nuclear protein was extracted for 30 min on ice, followed by 15 min of centrifugation in a microfuge. The supernatant was adjusted to a 50% final concentration of glycerol and stored at −20°C.

Topo II enzymatic activity was assayed by the ATP-dependent decatenation of kDNA [11]. The standard reaction mixture for the decatenation assay was 50 mmol/l Tris–HCl (pH 7.5), 120 mmol/l KCl, 10 mmol/l $MgCl_2$, 0.5 mmol/l dithiothreitol, 1 mmol/l ATP and 30 µg bovine serum albumin per ml. Decatenation of kDNA was carried out by incubating 5 µl of nuclear extract with 0.15 µg kDNA in a final volume of 25 µl of the standard reaction mixture for 15 min at 37°C. Reactions were terminated with 0.1-1 volume stop buffer (5% Sarkosyl, 0.025% bromophenol blue and 50% glycerol). Samples were then electrophoresed in 1% agarose in 89 mmol/l of Tris–borate and 2 mmol/l EDTA (pH 8.3) at 35 V for 4 h. To quantify the amount of decatenated DNA, photographic negatives of the ethidium bromide-stained agarose gels were densitometrically scanned.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from cells using Trizol according to manufacturer's instructions (Invitrogen). The reverse transcription was performed using the Superscript First-Strand Synthesis System (Invitrogen) with Oligo $d(T)_{18}$ primer. The primer used in this study for polymerase chain reaction (PCR) amplification was as follows: The Topo IIα primers [1] used were sense, 5′-TGTGGAGAA GCGGCTTGGTC-3′ (nt 91–71) and antisense, 5′-TAG TTACTAGAAGTTAGGAGCTG-3′ (nt 449–482). The MRP2 primers used were sense 5′-GCCAGATTGGCC CAGCAAA-3′ and antisense 5′-AATCTGACCACCGG CAGCCT-3′. Primers for GAPDH [12] used as controls for the reverse transcriptase (RT) reactions were sense 5′-CGGGAAGCTTGTCATCAATGG-3′ (nt 252–267) and antisense 5′-GGCAGTGAT GGCATGGACTG-3′ (nt 590–605). The PCR conditions were 94°C for 30 s, 50°C for 40 s and 72°C for 40 s, over 30 cycles. PCR products were visualized with ethidium bromide on 1% agarose gels.

Immunoblotting

Whole-cell extracts were obtained by lysis of cells in a 2 × sodium dodecyl sulfate (SDS)–polyacrylamide gel

electrophoresis sample buffer (125 mmol/l Tris–HCl, pH 6.8, 6% SDS, 10% glycerol, 10 mmol/l of 2-mercaptoethanol). Whole-cell extracts (30 or 50 μg of protein) were separated by electrophoresis on 8% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes in 25 mmol/l Tris–HCl (pH 8.3) containing 90 mmol/l glycine and 20% methanol for 2 h at 100 mA. The membranes were blocked with Tween Tris-buffered saline (TBS) with 0.1% Triton X-100 and 5% nonfat milk for 2–3 h at room temperature. Primary antibodies were diluted in a blocking solution (1 : 200 for Topo IIα, Sp1, Sp3, MRP2 and NF-YA, and 1 : 5000 for GAPDH). Membranes were incubated overnight at 4°C with the primary antibodies. After washing with TBS Tween-20 for 30 min, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The membranes were then washed for 1 h with TBS Tween-20. Bands were developed using chemiluminescent detection reagents according to the manufacturer's instructions, and signal detection and quantification were carried out with an ECL system (Amersham Biosciences, Piscataway, New Jersey, USA) and Image Quant analysis software (Quantity One; Bio-rad, Hercules, California, USA).

Statistical analysis

All assays were performed in triplicate. Data are expressed as the mean ± SD derivations of values. Statistical analyses were performed using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

The cytotoxic and chemosensitizing effects of BM-cyclin 1 in C-A120 cells

The cytotoxic and chemosensitizing effects of BM-cyclin 1 in C-A120 cells were determined using an MTT assay. The IC_{50} values were calculated and are listed in Table 1. After incubation with 1, 5 and 10 μg/ml BM-cyclin 1 for 96 h, the sensitivity to ADM was enhanced 2.1, 3.9 and 6.0 times, the sensitivity to VP-16 was enhanced by 1.7, 2.4 and 8.2 times, and the sensitivity to cisplatin was enhanced by 1.1, 1.2 and 1.7 times, respectively. Furthermore, we also studied the cytotoxicity to C-A120 cells of BM-cyclin 1. Cells were treated with various concentrations of BM-cyclin 1 (0.625, 1.25, 2.5, 5, 10, 20 and 40 μg) for 96 h. The results showed that the concentrations of BM-cyclin 1 used were low cytotoxic *in vitro* (Table 2). This indicated that the toxicity of BM-cyclin 1 is minimal at the concentrations required for the effective reversal of drug resistance.

Levels of topoisomerase IIα protein and mRNA

The expression levels of Topo IIα were analyzed by immunoblotting of whole-cell protein extracted from the cell lines. The Topo IIα protein level was 4-fold lower in C-A120 cells compared with parental KB cells. The Topo IIα protein level was increased approximately 2.2-fold in BM-cyclin 1-treated C-A120 cells compared with the C-A120 cells (Fig. 2). To determine whether the observed increase of the Topo IIα protein was due to increased levels of Topo IIα mRNA, total RNA was extracted from cell lines and analyzed by RT-PCR. The levels of Topo IIα mRNA in C-A120 cells were decreased to about 40% of those in the parental KB cells. The treated cells contained about 1.1-fold increased Topo IIα mRNA compared with the untreated C-A120 cells (Fig. 3).

The expression of Topo IIα transcription factors

As transcription factors implicated in the regulation of Topo IIα gene expression, the relative levels of Sp1, Sp3 and NF-YA protein were investigated in each group relative to GAPDH. In parallel with Topo IIα, C-A120 cell lines exhibited a significant decrease in Sp1 and NF-YA expression. The protein levels of Sp1 and NF-YA were 50 and 80% lower in C-A120 cells compared with parental KB cells. After treatment with BM-cyclin 1, the protein levels of Sp1 and NF-YA were increased approximately 0.27- and 0.48-fold, compared with the untreated C-A120 cells, respectively (Fig. 5). However, the expression of Sp3, an inhibitory member of Sp1 family, did not show any significant change (Fig. 5).

Topoisomerase II catalytic activity

The ability of Topo II to decatenate kDNA in the C-A120 cells was determined in nuclear extracts derived from KB,

Table 1 Effects of BM-cyclin1 on the cytotoxicity of ADM, VP-16 and cisplatin against C-A120 cell line

BM-cyclin1 (μg/ml)	IC_{50} (μg/ml)[a]						Fold reversal of MDR[b]		
	KB			C-A120			ADM	VP-16	Cisplatin
	ADM	VP-16	Cisplatin	ADM	VP-16	Cisplatin			
0	0.20 ± 0.01	0.90 ± 0.01	0.60 ± 0.01	2.80 ± 0.38	117.25 ± 6.90	1.80 ± 0.20			
1	0.20 ± 0.01	1.30 ± 0.01	0.60 ± 0.01	1.30 ± 0.23	68.10 ± 5.20	1.50 ± 0.20	2.1	1.7	1.1
5	0.20 ± 0.02	1.30 ± 0.01	0.60 ± 0.03	0.70 ± 0.02	49.59 ± 5.10	1.40 ± 0.30	3.9	2.4	1.2
10	0.10 ± 0.01	1.40 ± 0.01	0.50 ± 0.01	0.50 ± 0.03	14.37 ± 2.50	1.00 ± 0.10	6.0	8.2	1.7

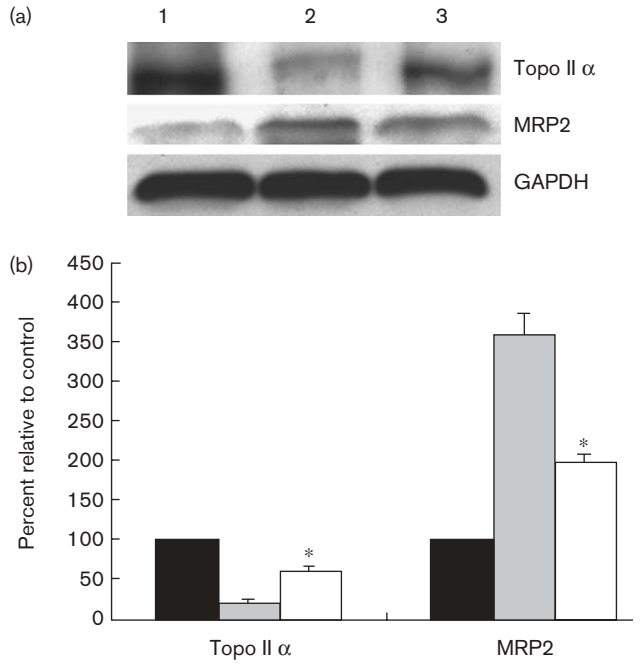
ADM, adriamycin; MDR, multidrug resistant; VP-16, etoposide.
[a]IC_{50} is the concentration of drug that reduced the cell survival to 50% of control. Each drug concentration was tested in triplicate.
[b]The reversal activity of target compounds was expressed as fold reversal (IC_{50} value for C-A120 cell with the reversing agents divided by IC_{50} value for C-A120 cell without the reversing agents).

Table 2 Sensitivity to BM-cyclin1 of C-A120 cells

BM-cyclin1 (μg/ml)	OD	Growth inhibition (%)	*P*
40	0.79 ± 0.06	12.34	<0.01
20	0.83 ± 0.06	7.12	<0.05
10	0.88 ± 0.03	1.67	>0.05
5.0	0.88 ± 0.05	1.62	>0.05
2.5	0.89 ± 0.06	1.56	>0.05
1.25	0.89 ± 0.02	1.53	>0.05
0.625	0.89 ± 0.01	0.13	>0.05
0	0.90 ± 0.03		

Cells were treated with various concentrations of BM-cyclin 1 for 96 h. Growth inhibition was assessed by MTT assays. The results reported the in-vitro cell proliferation as percentage of cell viability measured on control culture in the absence of drug. Each point represents the mean ± SDs of three experiments performed in duplicate. OD, optical density.

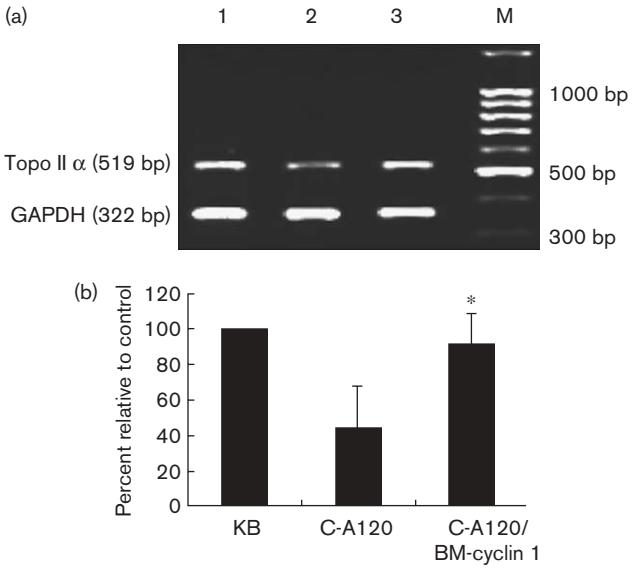
Fig. 2



(a) Immunoblots of whole-cell extracts with antibodies against Topo IIα and multidrug resistance protein 2 (MRP2). Lane 1: KB cells; lane 2: untreated C-A120 cells; lane 3: BM-cyclin1-treated C-A120 cells (10 μg/ml; for 96 h). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control in each assay. (b) Quantification of immunoblot data from at least three experiments. Black bar, KB cells; gray bar, untreated C-A120 cells; white bar, BM-cyclin1-treated C-A120 cells. The amount of each protein was calculated relative to GAPDH and normalized to the KB cell, which was arbitrarily set at 100%. Results are presented as mean ± SD. *$P < 0.01$ were obtained by comparing the relative amounts of each protein in BM-cyclin1-treated cells with the untreated control cells using Student's *t*-test.

C-A120 and BM-cyclin 1-treated C-A120 cells. Topo II decatenation activity (per identical amounts of nuclear extract proteins) was 30% higher in BM-cyclin 1-treated C-A120 cells, compared with C-A120 cells, as determined by a comparison of the banding intensities of the mini-circles (Fig. 6). This finding is consistent with the fact that Topo IIα protein level is increased in the treated cells.

Fig. 3



(a) Effect of BM-cyclin1 on Topo IIα mRNA in C-A120 cells. The expression of Topo IIα mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). BM-cylin1 significantly increased the Topo IIα mRNA expression level. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for an internal control. Lane 1: PCR products using complementary DNA (cDNA) from KB RNA; lane 2: PCR products using cDNA from C-A120 RNA; lane 3: PCR products using cDNA from BM-cylin1-treated (10 μg/ml; for 96 h) C-A120 RNA; lane M, 100-bp DNA ladder (Invitrogen Life Technologies). (b) Quantification of PCR data from at least three experiments. The amount of each mRNA was calculated relative to GAPDH and normalized to the KB cell, which was arbitrarily set at 100%. Results are presented as mean ± SD. *$P < 0.01$ were obtained by comparing the relative amounts of mRNA in BM-cyclin1-treated cells with the untreated control cells using Student's *t*-test.

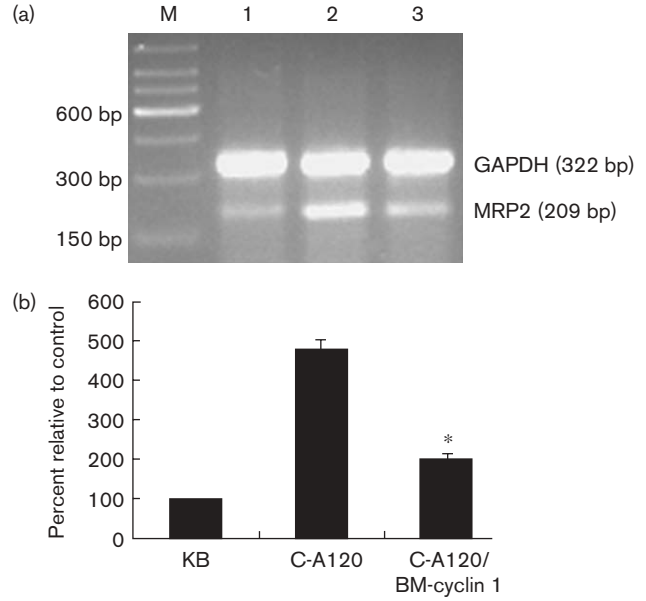
Expression of multidrug resistance protein 2

As one of the factors of MDR, the relative protein level of MRP2 was determined by immunoblotting. The result showed that the protein level of MRP2 was 2.6-fold higher in C-A120 cells compared with parental KB cells. After treatment with BM-cyclin 1 for 96 h, the protein level was decreased approximately 80% compared with the C-A120 cells (Fig. 2). To determine whether the observed decrease of the MRP2 protein was due to decreased level of MRP2 mRNA, total RNA was extracted from cell lines and analyzed by RT-PCR. The levels of MRP2 mRNA in C-A120 cells were increased to about five times those in the parental KB cells. The treated cells contained about 2.4-fold decreased MRP2 mRNA compared with the untreated C-A120 cells (Fig. 4).

Discussion

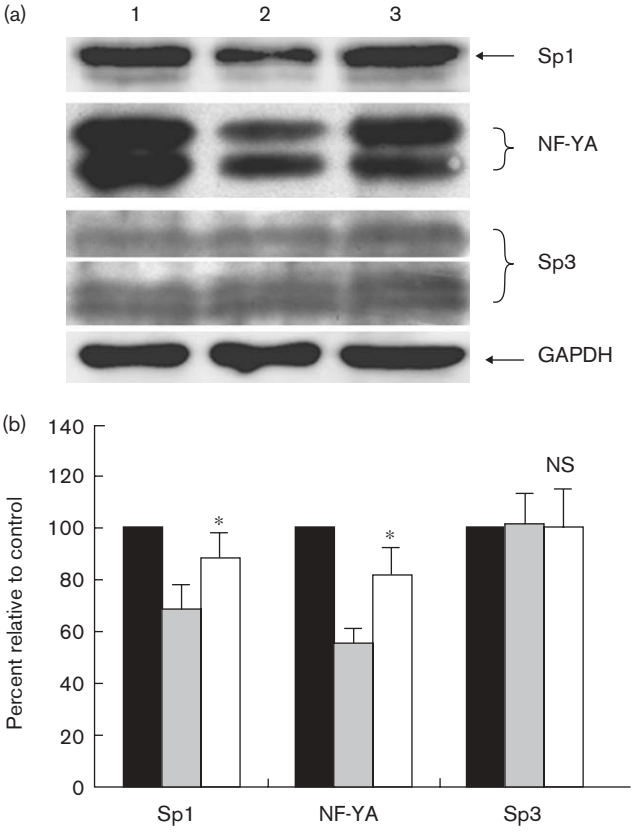
BM-cyclin 1, macrolide tiamulin, is one of the pleuromutilin derivatives. It has been widely used as a potent antimycoplasma agent [9,13]. The antibiotic tiamulin targets the 50S subunit of the bacterial ribosome and interacts at the peptidyl transferase center [14,15]. Interestingly, our studies have proved that BM-cyclin 1

Fig. 4



(a) Effect of BM-cyclin1 on multidrug resistance protein 2 (MRP2) mRNA in C-A120 cells. The expression of MRP2 mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). BM-cyclin1 significantly suppressed the MRP2 mRNA expression level. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Lane 1: PCR products using complementary DNA (cDNA) from KB RNA; Lane 2: PCR products using cDNA from C-A120 RNA; lane 3: PCR products using cDNA from BM-cylin1-treated C-A120 RNA (10 μg/ml; for 96 h); lane M, 150-bp DNA ladder (Invitrogen Life Technologies). (b) Quantification of PCR data from at least three experiments. The amount of each mRNA was calculated relative to GAPDH and normalized to the KB cell, which was arbitrarily set at 100%. $*P<0.01$ were obtained by comparing the relative amounts of mRNA in BM-cyclin1-treated cells with the untreated control cells using Student's *t*-test.

Fig. 5



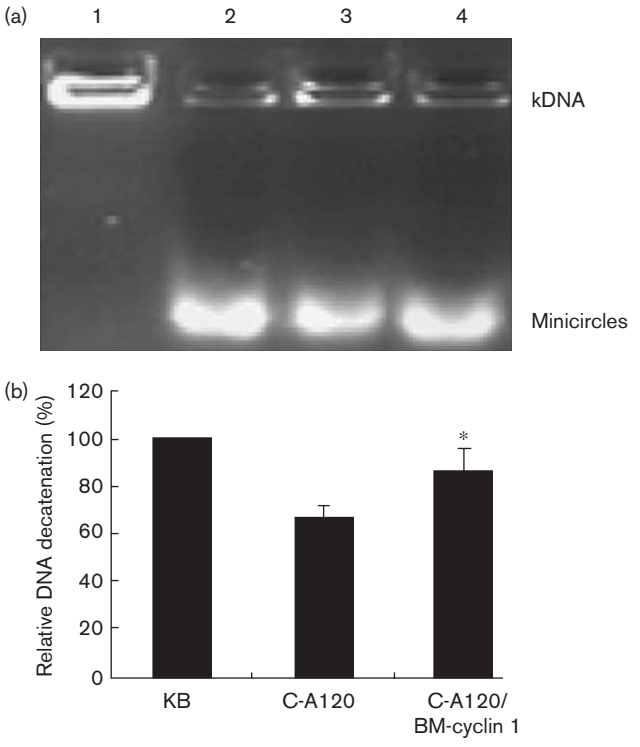
(a) Immunoblots of whole-cell extracts with antibodies against Sp1, NF-YA and Sp3. Lane 1, KB cells; lane 2, untreated C-A120 cells; lane 3, BM-cyclin1-treated C-A120 cells (10 μg/ml; for 96 h). GAPDH was included as a control in each assay. (b) Quantification of immunoblot data from at least three experiments. Black bar, KB cells; gray bar, untreated C-A120 cells; white bar, BM-cyclin1-treated C-A120 cells. The amount of each protein was calculated relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized to the KB cell, which was arbitrarily set at 100%. Results are presented as mean ± SD. NS, $P>0.05$, $*P<0.01$ were obtained by comparing the relative amounts of each protein in BM-cyclin1-treated cells with the untreated control cells using Student's *t*-test.

exerts its reversal effects markedly on MDR. In this study, we reported for the first time that BM-cyclin 1 effectively reversed drug resistance and increased the expression of Topo IIα at both the protein and the mRNA levels in C-A120 cells. Thus, BM-cyclin 1 might be a new reversing agent for Topo IIα-mediated resistance. It has been suggested that the C-A120 cells are non-P-gp-mediated resistance cells. The C-A120 cells showed much higher tolerance to ADM, VP-16 and cisplatin, compared with their parental KB cells, from drug-sensitive cell lines [4].

Our cytotoxicity study revealed that after incubation with 10 μg/ml BM-cyclin 1 for 96 h, the sensitivity of C-A120 cells to ADM, VP-16 and cisplatin was enhanced 6.0, 8.2 and 1.7 times, respectively. In addition, the concentrations of BM-cyclin 1 used here were of low cytotoxicity *in vitro* (Table 1). These results suggested that BM-cyclin 1 could effectively reverse drug resistance in C-A120 cells. Resistance to agents that target Topo II is a major problem in cancer chemotherapy. In addition to the classical MDR, which is due to the overexpression of the MDR transporter (e.g. P-gp and MRPs), atypical MDR (at-MDR) has been described; it is associated with altered Topo II activity owing either to its mutation or to the decreased amount of enzyme expression [16]. Down-regulation of Topo IIα is associated with the drug-resistant phenotype in many cell lines and cancer tissues [3,17]. With respect to the Topo II poisons, the level or activity of the Topo IIα enzyme can determine the amount of drug-induced DNA damage that occurs and in turn might determine the cytotoxicity of the treatment [2].

Earlier reports have suggested that the Topo II activity is decreased in drug-resistant cells [11,18,19]. The cross-resistance pattern of C-A120 cells to the Topo II targeted drugs suggests that quantitative and/or qualitative alterations of the Topo II catalytic activity might be

Fig. 6



Topo II activity detected in nuclear extracts from KB, C-A120 and BM-cyclin1-treated C-A120 cells. Topo II activity in nuclear extracts from cells was measured by the decatenation assay of kinetoplast DNA (kDNA) as described in Materials and methods. (a) As shown in lane 1, control, 0.15 μg of kDNA without nuclear extract protein, the high-molecular-weight kDNA did not move from the loading well origin; the substrate, 0.15 μg of kDNA, was incubated for 30 min with nuclear extracts as follows. Lane 2: nuclear extract protein from KB cells; lane 3: nuclear extract protein from C-A120 cells and lane 4: nuclear extract protein from BM-cycin1-treated C-A120 cells (10 μg/ml; for 96 h). (b) Quantification of Topo II decatenation data from at least three experiments. The percentage of decatenation was calculated relative to KB cells and normalized to the KB, which was arbitrarily set at 100%. $*P<0.01$ compared with the untreated control cells.

involved in the MDR phenotype. In this study, the expression of Topo IIα in treated C-A120 cells compared with the untreated C-A120 cells increased significantly at both the protein and the mRNA levels (Figs 2 and 3). These results suggest that the mechanism of BM-cycin1 to reverse MDR might be activated by the increase in the Topo IIα protein level.

Topo II catalytic activity in BM-cyclin 1-treated C-A120 cells was increased by 30% compared with untreated cells, as measured by the decatenation of kDNA (Fig. 6). The discovery is consistent with the finding that the Topo IIα protein level is increased in the treated cells. This indicated that BM-cyclin 1 enhances the Topo IIα level and activity, thus resulting in the reversal of MDR in C-A120 cells. The finding that C-A120 cells contained a reduced level of Topo IIα mRNA indicates that either the transcriptional or the posttranscriptional regulation could account for the decrease in Topo IIα protein and activity. Although Topo IIα expression can be regulated at multiple levels, it has been implied that the reduced Topo II expression is mostly due to its decreased transcript level [20].

The human Topo IIα promoter has been shown to contain two GC boxes, five inverted CCAAT boxes (ICB1–5) and one activating transcription factor element, among the putative transcription factor binding sites identified [21]. The ubiquitous transcription factors NF-YA, Sp1 and Sp3 have been implicated in the regulation of the Topo IIα promoter [22–24]. NF-YA can bind to ICB1–4, although the affinity for binding at each element varies. NF-YA has been implicated as an activator when bound at ICB1. Earlier reports showed that a reduction in NF-YA binding to ICB1–4 has been implicated in the downregulation of Topo IIα promoter in drug-resistance cells [20]. Sp1 has been identified as a potential activator of the basal Topo IIα promoter activity, and both Sp1 and Sp3 have been shown to bind to GC1 and GC2 [25]. Sp3 might act as a repressor to inhibit Sp1-dependent transcription [22]; hence, the absolute levels of Sp1 and Sp3 or their nuclear ratio might be responsible for the differences in promoter binding and target gene expression [26].

An analysis of the expression levels of various transcription factors showed that BM-cyclin 1 treatment significantly enhanced the expression of Topo IIα transcription factors Sp1 and NF-YA in C-A120 cells (Fig. 5). This result is consistent with the change of Topo IIα protein. Although there was no significant change of Sp3 level in each group, the ratio Sp1/Sp3 increased. Our data suggest that the transcriptional upregulation caused by BM-cyclin 1 contributes to the increased expression of Topo IIα protein and might be associated with the reversal effect of the resistance in C-A120 cells.

We also observed the expression of MRP2 in this study. The human MRP2, known as the canalicular multispecific organic anion transporter, belongs to the MRP family. It functions as an energy-dependent (ATP-dependent) pump, exporting toxic substances from the cell, thus decreasing drug accumulation within the cells. The functional activities of MRP2 and P-gp transporters are different; the latter functions as a drug-effluxing pump, whereas MRP needs cellular glutathione. The protein is one of the transporters of glutathione conjugates (the so-called GS-X pumps). MRP2 has been found to be overexpressed in a number of cisplatin-resistant cell lines [27,28]. Earlier reports also suggested that the protein level of MRP2 in C-A120 cells was overexpressed compared with that in drug-sensitive KB cells [5]. In this study, expression analyses revealed that MRP2 was significantly repressed in BM-cyclin 1-treated C-A120 cells. After treating the cells with BM-cyclin 1 for

96 h, compared with the untreated C-A120 cells, the expression of MRP2 in treated C-A120 cells decreased significantly at both the protein and the mRNA levels (Figs 2 and 4). These results suggested that the BM-cyclin 1 showed reversal effects on the MDR phenotype, which might be consistent with the repressed expression of MRP2.

Our results indicated that the reversal activity of BM-cyclin 1 on C-A120 drug resistance was associated with the increased Topo IIα catalytic activity and protein levels. Transcriptional upregulation in treated cells resulted in the increased expression of Topo IIα protein. Furthermore, suppressing the expression of MRP2 by BM-cyclin 1 might be another mechanism to reverse MDR in C-A120 cells.

In summary, BM-cyclin 1 might be a potential MDR-reversal agent against non-P-gp-mediated MDR tumor cells that are resistant to ADM, VP-16 and cisplatin. Although this study demonstrated that BM-cyclin 1 is an effective reversal agent *in vitro*, experimentation *in vivo* is required to determine the potential MDR reversal effect for non-P-gp-mediated MDR.

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