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Inhibition of microtubule polymerization by 3-bromopropionylamino benzoylurea (JIMB01), a new cancericidal tubulin ligand

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Abstract

3-Bromopropionylamino benzoylurea (JIMB01) is a small molecular weight compound (MW 313) that has been synthesized in our laboratory. This compound showed antiproliferative activities in a panel of thirteen human tumor cell lines with IC_{50} values in the range of 0.25 to 0.51 μM for leukemia and lymphoma cell lines and 0.33 to 9.26 μM for solid tumor cell lines. The primary action of JIMB01 is to inhibit microtubule polymerization but not depolymerization. A 4 μM concentration of the compound caused a complete inhibition of microtubule assembly in a cell-free reaction. An increase in the number of human hepatocarcinoma cells blocked in the M-phase was detected 12 hr after exposure to JIMB01. The kinase activity of cyclin B1, which is responsible for the G_2/M transition, was increased accordingly. Bcl-2 phosphorylation became visible, in a western blot, within 6 hr in hepatocarcinoma cells treated with JIMB01 at 0.8 μM or higher. JIMB01-induced apoptosis in liver cancer cells was confirmed by morphological methods, flow cytometry, as well as DNA gel electrophoresis, which clearly demonstrated DNA degradation in the form of a multiple-unit DNA ladder. Furthermore, *in vivo* experiments using nude mice showed that intraperitoneal injection of JIMB01 at 15 mg/kg (with seven injections at 4-day intervals) significantly inhibited the growth of a human hepatocarcinoma (BEL-7402) by 66% in tumor volume ($P = 0.01$), at least compatible to the inhibition by vincristine (43% inhibition), indicating good bioavailability of the compound in the circulation. Side-effects of the compound were not observed, and the body weight of the treated mice remained stable during the 4-week treatment. Since JIMB01 is a small compound, targets a specific molecule in tumor cells, and has promising activity against human hepatocarcinoma *in vivo*, we believe JIMB01 merits consideration for further investigation.

Keywords: JIMB01; Antimicrotubule; Apoptosis; Chemotherapy; Hepatocarcinoma

1. Introduction

Two distinct antimicrotubule mechanisms have been recognized among the agents that interfere with the natural cycle of microtubule assembly–disassembly: (a) inhibiting the polymerization of tubulin; and (b) promoting the polymerization of tubulin polymers to form abnormal and excessively stable microtubule structures [1]. The first FDA-approved microtubule agents were vinca alkaloids, which inhibit the assembly of microtubule spindles from the free tubulin pool [2,3] and show a therapeutic effect in cancer patients [1,4]. In contrast, paclitaxel promotes the assembly of microtubules *in vitro* and *in vivo* [5,6]. After the approval of paclitaxel for the treatment of ovarian and breast cancer in patients [7,8], microtubule-active agents have become increasingly attractive. Compounds in this category, such as vinblastine, vincristine, podophyllotoxin, paclitaxel, and docetacel, have several common features. First, these drugs are all natural complexes from plants (or mimic synthetic variants); second, they are relatively large molecules, therefore, difficult to synthesize *de novo*; and third, they are all substrates of P-gp, and cause drug resistance [4,9,10]. Attempts to discover small tubulin

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Abbreviations: DTT, dithiothreitol; FDA, Federal Drug Administration; HRP, horseradish peroxidase; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBL, peripheral blood lymphocyte(s); P-gp, P-glycoprotein; PMSF, phenylmethylsulfonyl fluoride; and RT, room temperature.

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ligands with novel chemical structures as well as cancer therapeutic effects continue [11–14].

We have been searching for small molecular tubulin ligands that can be easily synthesized and that can specifically interrupt microtubular dynamics with a view to developing them as anticancer drugs [11,14]. Optimization of a series of compounds resulted in 3-bromopropionylamino benzoylurea (JIMB01), which was selected for further investigation because it had greater stability than 3-iodoacetamido benzoylurea [11] and stronger activity on microtubules than 3-bromoacetamido benzoic acid ethyl ester [14]. JIMB01 interferes with the assembly of microtubules from free tubulin in tumor cells, blocks the cell cycle at the M-phase, causes apoptotic cell death in a variety of cancer types, and has shown promising therapeutic efficacy in nude mice bearing human hepatocarcinomas. This paper reports our observations *in vitro* and *in vivo* using this compound.

2. Materials and methods

2.1. Reagents

JIMB01 was synthesized in our laboratory. It has a molecular weight of 313 Da, and its structure (Fig. 1) was confirmed by mass spectrometry and NMR. A 32 mM stock solution of the compound was prepared in a mixture of *N*,*N*-dimethylacetamide, propylene glycol, and Tween-80 (1:2:1, by vol.) and was diluted in medium prior to use. Paclitaxel, vinblastine sulfate, and vincristine (Aldrich Chemical Co.) were dissolved in DMSO before use and diluted in medium. Equal volumes of solvents were used as controls.

2.2. Cell lines

All the tumor lines were from human malignant tumors and are listed in Table 1. Daudi/MDR [14] and DND-1A cells were provided by Dr. T. Ohnuma (Mount Sinai School of Medicine); the human hepatocarcinoma lines BEL-7402, QGY-7701, and SMMC-7721 were from the Cancer Institute, Chinese Academy of Medical Sciences; the other cell lines were obtained from the American Type Culture Collection. All of the cell lines were cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (250 units/mL), and streptomycin (250 units/mL),

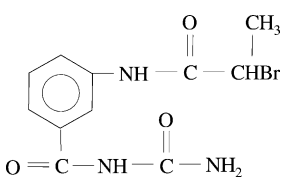

Fig. 1. Chemical structure of 3-bromopropionylamino benzoylurea (JIMB01).

Table 1
Antiproliferative activities of JIMB01 in human tumor cell lines

Human cell line	Tumor type	IC_{50} (μM)
CEM	T-cell leukemia	0.25 ± 0.01
U937	Histiocytic leukemia	0.25 ± 0.02
NB40	Promyelocytic leukemia	0.33 ± 0.05
HL60	Promyelocytic leukemia	0.3 ± 0.04
Daudi/wt	B-cell lymphoma	0.51 ± 0.03
Daudi/MDR	B-cell lymphoma (resist.)	0.45 ± 0.08
HCT-116	Colon cancer	4.73 ± 0.2
BT-483	Breast cancer	0.33 ± 0.04
DND-1A	Melanoma	0.6 ± 0.1
BEL-7402	Hepatocarcinoma	0.63 ± 0.02
HepG2	Hepatocarcinoma	0.48 ± 0.01
QGY-7701	Hepatocarcinoma	1.04 ± 0.16
SMMC-7721	Hepatocarcinoma	9.26 ± 0.21
PBL (PHA)[a]	Normal lymphocytes	8.96 ± 1.3
HU-VEC	Normal endothelial cells	10.88 ± 1.6

Values are means ± SD, N = 3; evaluated with the MTT assay.
[a] PBL (PHA), peripheral blood lymphocytes stimulated with PHA at 37° for 24 hr.

with the exception of BT-483, which was grown in Dulbecco's Modified Eagle's Medium. Human PBL were isolated from the whole blood of healthy individuals by the Ficoll-Hypaque gradient method [14]. Isolated PBL were maintained in RPMI-1640 in the presence of 10% FBS at 37°. Cells in exponential growth were used.

2.3. Determination of IC_{50} values

Cells were seeded into 96-well microplates (Falcon) at 2.5×10^4/well, followed by treatment with JIMB01 at concentrations between 0 and 16 μM for 72 hr at 37°. Cell viability was assessed by MTT staining [15]. Cancericidal activity was determined in duplicates, and each experiment was repeated three times under identical conditions. The IC_{50} was defined as the drug concentration that induced 50% cellular death in comparison with untreated controls and was calculated by non-linear regression analyses.

2.4. Morphology

Cell samples on slides were prepared in a Cytospin centrifuge (LTP-C, Experimental Apparatus Factory, CAMMS) at 700 *g* for 5 min at RT. For Giemsa staining, the slides were air-dried, fixed in methanol, and stained in Giemsa at RT for 15 min. Cells in the mitotic phase were recognized by the appearance of dispersed chromosomes in the cytoplasm, and by the disappearance of the nuclear membrane. Apoptotic cells were identified using previously defined criteria, i.e. cell shrinkage, chromatin condensation, and fragmentation of the nucleus into discrete masses [16]. For the fluorescent staining, cell samples were resuspended in 100 μL of Hoechst 33258 solution (100 μmol/L, Aldrich) followed by a 30-min incubation at 37°. Apoptotic cells were observed under UV illumination on a Nikon fluorescent microscope.

2.5. Cell cycle

The cell cycle was analyzed by a method reported previously [11]. Briefly, 5×10^6 cells were resuspended in 70% ethanol for at least 12 hr at 4°. After washing with PBS, the cells were treated with RNase A (50 μg/mL, Sigma) at 37° for 30 min, and then exposed to propidium iodide at a final concentration of 50 μg/mL at 4° for 30 min. Cells were analyzed in a flow cytometer (FACS 420, Becton Dickinson).

2.6. Detection of kinase activity

Cells exposed to JIMB01 were lysed with 0.5 mL of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1 mM NaF, 1 mM DTT, 1 mM PMSF, 1% Nonidet P-40, and 10 μg/mL of aprotinin and leupeptin. The kinase reaction was started by the addition of 30 μL of kinase reaction buffer containing 100 μM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 5 mg histone H1 (Boehringer Mannheim), 1 μM ATP, and 1 μCi [^{32}P]r-ATP (Amersham) at 30° for 15 min. The reaction was terminated by the addition of 4× Laemmli sample buffer (containing 62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.05% bromophenol blue) and resolved by 10% SDS–PAGE. The kinase activity was determined by either autoradiography or measuring radioactivity incorporated into histone H1 protein.

2.7. Inhibition assay of microtubule assembly and disassembly

Purified tubulin from calf brain was purchased from the Sigma Chemical Co. The effect of JIMB01 on the microtubule assembly–disassembly process was determined using the conditions recommended by the vendor. For assembly inhibition, 100 μL of tubulin solution (500–600 μg protein/mL) was mixed gently with 400 μL of reaction buffer containing 0.1 M 2-[*N*-morpholino]ethane sulfonic acid (MES), 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 2.5 M glycerol at 37°. Then JIMB01, paclitaxel, or vinblastine was added to each sample cuvet. After adding GTP to each sample to a final concentration of 1 mM, the microtubule assembly process was monitored by measuring the change of O.D. every 5 min at 350 nm on a spectrophotometer (Pharmacia LKB). Assembly was complete within 40 min at 37°. For disassembly inhibition, JIMB01, paclitaxel, or vinblastine was added to a cuvet with repolymerized microtubules according to the method described above, and incubated in melting ice. Changes of O.D. were monitored at 350 nm for 30 min until the O.D. values in the controls returned to the starting level, i.e. completion of the assembly–disassembly cycle. Solvent solutions were used as controls and had no effect on either the assembly or disassembly process.

2.8. Detection of Bcl-2 phosphorylation

The method has been described previously [17]. Tumor cells were treated with different concentrations of JIMB01 over the time range of 2 to 24 hr. Aliquots of cells were taken and lysed with lysis buffer containing 50 mM Tris–HCl (pH 7.4), 0.1% Triton X-100, 1% SDS, 250 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 1 mM PMSF, 10 μg/mL of leupeptin, and 10 μg/mL of aprotinin. The protein concentration was determined with the Bradford method. Equal amounts of lysate were subjected to electrophoresis using 0.1% SDS, 10% polyacrylamide gels. The gels were blotted onto nitrocellulose membranes using a semi-dry transfer cell (Bio-Rad). After blocking with 5% non-fat milk in TBST buffer (100 mM Tris–Cl, pH 7.5, 0.9% NaCl, 0.1% Tween-20) at RT for 1 hr, Bcl-2 protein was probed with anti-Bcl-2 monoclonal antibody (Santa Cruz Biotech, Inc.), followed by goat anti-mouse HRP. The signals were detected by enhanced chemiluminescence (ECL).

2.9. DNA fragmentation

DNA was extracted from the cells by a method reported previously [18]. Briefly, after washing with PBS, cells were treated in 1 mL of lysis buffer (10 mM Tris–HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA, pH 8.0, and 5% SDS) containing 1 μg/mL of protease K for 1 hr at 50°. The resulting products were extracted first with phenol, then with a mixture of chloroform:isoamyl alcohol (24:1; v/v), and finally precipitated with ethanol followed by centrifugation at 10,000 *g* for 45 min at 4° (PM180R, ALC Ltd.). The ethanol was removed, and the dry nucleic acid pellet was resuspended in 10 mM Tris–HCl/1 mM EDTA solution. DNA content was determined spectrophotometrically by measuring absorption at 260 nm (A_{260}). All samples had an $A_{260}/A_{280} \geq 2$. The DNA samples were then treated with RNase (Sigma) at a final concentration of 0.1 μg/μL for 20 min at 37° before loading the gel. The DNA sample (4 μg) was analyzed in a 1.5% horizontal agarose gel prepared with 1% ethidium bromide. Electrophoresis was performed at 35 V for 5 hr, and the DNA migration was visualized under UV light.

2.10. Anticancer effect in vivo

Nude mice (male, 6–7 weeks of age, and 16–20 g in weight) were obtained from the Experimental Animal Center, Chinese Academy of Medical Sciences, and used for human hepatocarcinoma (BEL-7402) xenografts. All tumor tissue grafts were processed carefully to remove the connective tissues and blood clots prior to inoculation. Experimental mice received subcutaneous trocar grafts of a human hepatocarcinoma (4 mm^3/mouse) on their backs. One week later, tumor-bearing nude mice were divided randomly into solvent control and treatment cages with 8

mice per group. JIMB01 at 5, 10, or 15 mg/kg was given i.p. on day 8 post-implantation. The regimens were continued with another six injections at 4-day intervals. Therapeutic response was monitored by measuring tumor volume every week until the tumor volume of the controls reached a size greater than 2000 mm^3, the criterion for euthanasizing the animals [19]. Two perpendicular tumor measurements, width and length, were obtained with calipers, followed by calculation using the formula: tumor volume = length × $width^2$ × 0.52 [20]. Student's *t*-test was used for the analysis of tumor volume between groups.

3. Results

3.1. In vitro cancericidal activity

As shown in Table 1, antiproliferative activity of JIMB01 *in vitro* was tested in thirteen human tumor cell lines representing eight different types of cancer. The IC_{50} values for JIMB01 were in the range of 0.33 to 9.26 μM for solid tumor lines and 0.25 to 0.51 μM for leukemia and lymphoma cell lines. The most sensitive cell lines were CEM and U937 (IC_{50} = 0.25 μM for both), 35-fold more susceptible than phytohemagglutinin (PHA)-stimulated PBL and 44-fold than HU-VEC. The least sensitive one was a liver cancer cell line, SMMC-7721 (IC_{50} = 9.26 μM). A significant diversity of the sensitivity to JIMB01 was noticed in the four human hepatocarcinoma cell lines tested in this study, among which HepG2 and BEL-7402 were much more susceptible than SMMC-7721. The IC_{90} of JIMB01 in BEL-7402 cells was 3.2 μM. This was the concentration used for the flow cytometry and morphology experiments presented below. JIMB01 seemed not to be a substrate of P-gp because the susceptibility to JIMB01 of Daudi/wt and Daudi/MDR cells was essentially the same.

3.2. Mitotic block in cell cycle

The cell cycle of JIMB01-treated and untreated hepatocarcinoma BEL-7402 cells was analyzed. Untreated cells showed a classical pattern of proliferating cells proportionally distributed in G_0/G_1 (57%), S (33.1%), and G_2/M (3.5%) phases (Table 2). Treatment with JIMB01 induced a major shift from the G_0/G_1 to the G_2/M phase. The G_2/M phase peak was elevated within 12 hr of treatment, and increased continuously for 24 hr after which the proportion of apoptotic cells increased. By 24 hr, 57.5% of the cells were in the G_2/M phase and by 48 hr 81.5% of the cells were apoptotic. Over this 48-hr period, there was a coincident reduction in the proportion of cells in the G_0/G_1 phase, from 57 to 1.5% (Table 2 and Fig. 2). Other cell lines (HepG2 and HCT-116) treated with JIMB01 showed a similar efficacy of mitotic arrest (not shown).

Table 2
Arresting of human hepatocarcinoma BEL-7402 cells at the G_2/M phase by JIMB01

Treatment	Hours of treatment	Cell distribution (%)[a]			
		G_0/G_1	S	G_2/M	Apoptosis
JIMB01 (3.2 μM)	0	57	33.1	3.5	6.4
	12	44.7	40.7	8.4	6.2
	24	3.3	33	57.5	6.2
	36	5.5	14.9	53.7	25.8
	48	1.5	4.5	12.5	81.5

[a] See Fig. 2 for the original results. Cells and cell fragments with a DNA content less than G_0/G_1 were defined as apoptotic.

3.3. Activity of cyclin B1

To further prove the M-phase arrest by JIMB01, kinase activity of mitotic cyclin B1 was analyzed in JIMB01-treated BEL-7402 cells (Fig. 3). Kinase activity of cyclin B1 in BEL-7402 cells increased when the concentration of JIMB01 was 1.6 μM and greater and became detectable within 12 hr of treatment. Kinase activities of cyclin A and D1, which are relevant to S and G_1 phases, remained unchanged after exposure to JIMB01 (not shown).

3.4. Apoptosis in hepatocarcinoma cells

The apoptotic effect of JIMB01 on liver cancer cells was studied with microscopy and gel electrophoresis. Three different human liver cancer cell lines treated with JIMB01 for 24 hr had similar apoptotic characteristics (Fig. 4). The effect of JIMB01 treatment was a spreading of the M-phase

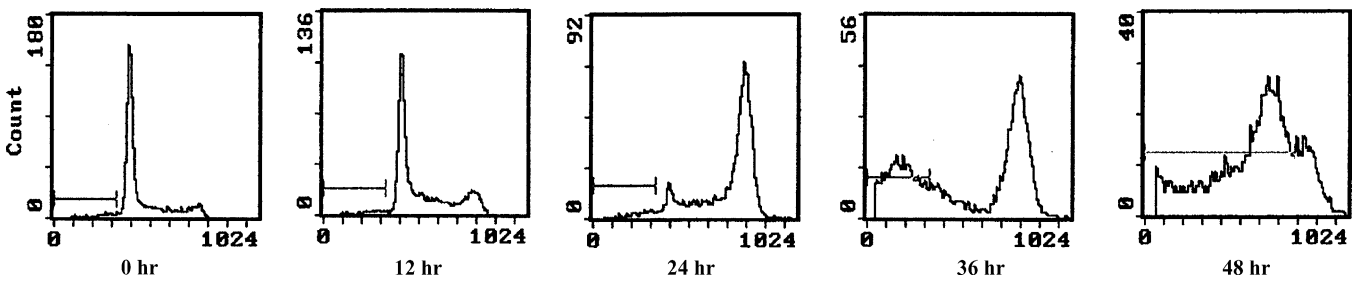

Fig. 2. Arresting cell division at the G_2/M phase in human hepatocarcinoma cells treated with JIMB01. Cell cycle analyses of BEL-7402 cells treated with JIMB01 (3.2 μM) was performed by flow cytometry at 0, 12, 24, 36, and 48 hr after treatment, and compared with that of untreated BEL-7402 cells. Most of the treated cells were accumulated at the G_2/M phase 24 hr after treatment.

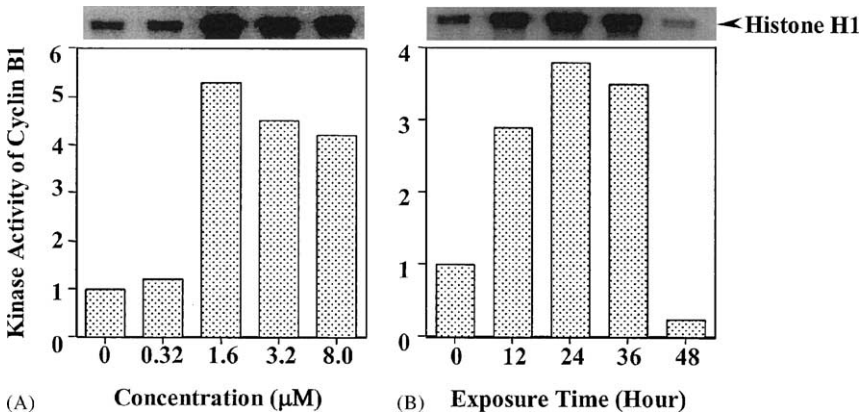

Fig. 3. Effect of JIMB01 on cyclin B1 kinase activity. BEL-7402 cells were exposed to various concentrations of JIMB01 for 24 hr (A), or to 3.2 μM JIMB01 for various periods of time (B). The kinase activity of cyclin B1 was measured by autoradiography of ^{32}P incorporation into histone H1 (the top of panels A and B); relative kinase activity was determined by measuring ^{32}P incorporation, accordingly (the bottom of panels A and B). The experiment was repeated twice.

chromosomes (Fig. 4B, center), and then the formation of condensed chromatin into discreted masses (Fig. 4). After staining the JIMB01-treated BEL-7402 cells with Hoechst 33258, fluorescence of the intracellular nuclear fragments was clearly seen (Fig. 5). To confirm the morphological findings, DNA was extracted from the treated BEL-7402 cells. Gel analyses (Fig. 6) exhibited a classical internucleosomal DNA fragmentation after 48 hr of treatment.

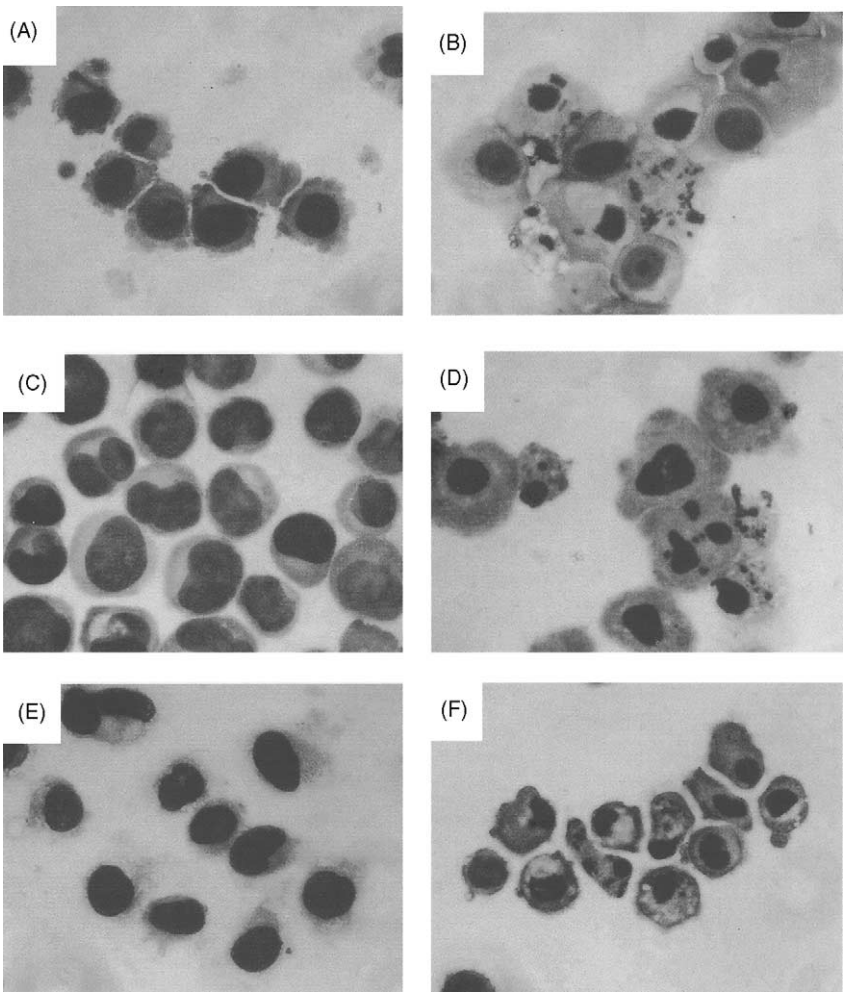

Fig. 4. M-phase block and apoptosis in human hepatocarcinoma cells treated with JIMB01 for 24 hr. (A) Untreated HepG2 cells; (B) treated HepG2 cells (0.8 μM); (C) untreated BEL-7402 cells; (D) treated BEL-7402 cells (3.2 μM); (E) untreated QGY-7701 cells; (F) treated QGY-7701 cells (3.2 μM). Giemsa staining (400×).

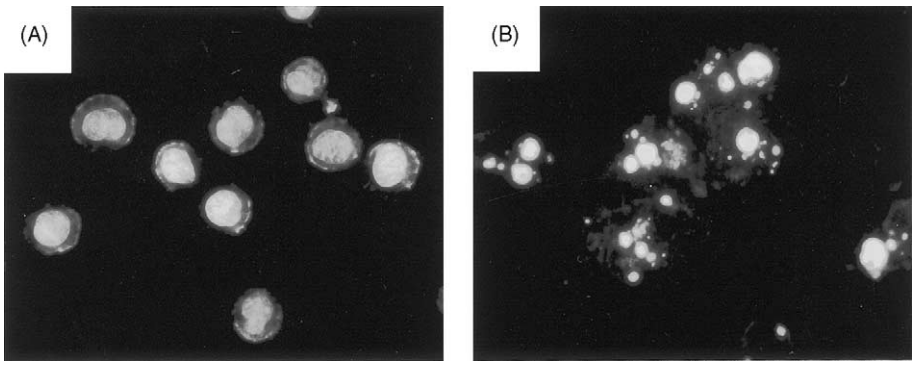

Fig. 5. Apoptotic nuclear degradation by JIMB01 in human hepatocarcinoma cells. (A) Untreated BEL-7402 cells; (B) treated BEL-7402 cells (3.2 μM). The cells were treated for 48 hr, and stained with Hoechst 33258 as described in "Section 2" (400×).

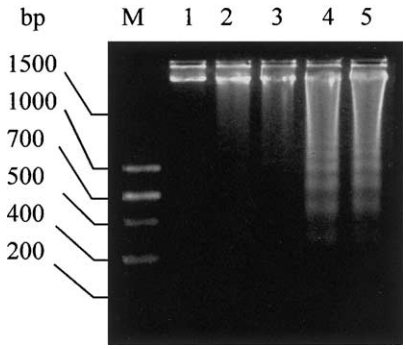

Fig. 6. Apoptotic DNA ladder in BEL-7402 cells exposed to JIMB01. BEL-7402 cells were treated with JIMB01 at 0.08, 0.32, 0.8, and 3.2 μM, respectively (lanes 2–5), for 48 hr. A DNA ladder, characteristic of apoptosis, was clearly visualized at 0.8 μM or higher. Lane 1 is the untreated control, and lane M is the molecular marker.

Similar results were obtained in other tumor cells (not shown). The spreading of the chromosomes was identified most clearly in leukemia/lymphoma cells, such as CEM and Daudi (not shown).

3.5. Effects on the microtubule assembly–disassembly

Since the microtubules are the major apparatus responsible for M-phase division, it is reasonable to consider the compound a ligand for tubulin. The effects of JIMB01, vinblastine, and paclitaxel on the kinetics of the microtubule assembly–disassembly cycle are shown in Fig. 7. Untreated tubulins exhibited a temperature-dependent assembly–disassembly cycle as long as Mg^{2+} and GTP were present. JIMB01 interrupted the assembly process, but not the disassembly. A complete inhibition of assembly was found when JIMB01 was used at a concentration of 4 μM, whereas microtubular disassembly was not influenced even when the JIMB01 concentration was 32 μM. Vinblastine had a mechanism of action similar to that of JIMB01. However, paclitaxel exhibited an inhibitory effect only on the disassembly process. In addition, we used transmission electron microscopy to visualize the microtubule structure in samples treated with JIMB01, and the results were negative (not shown).

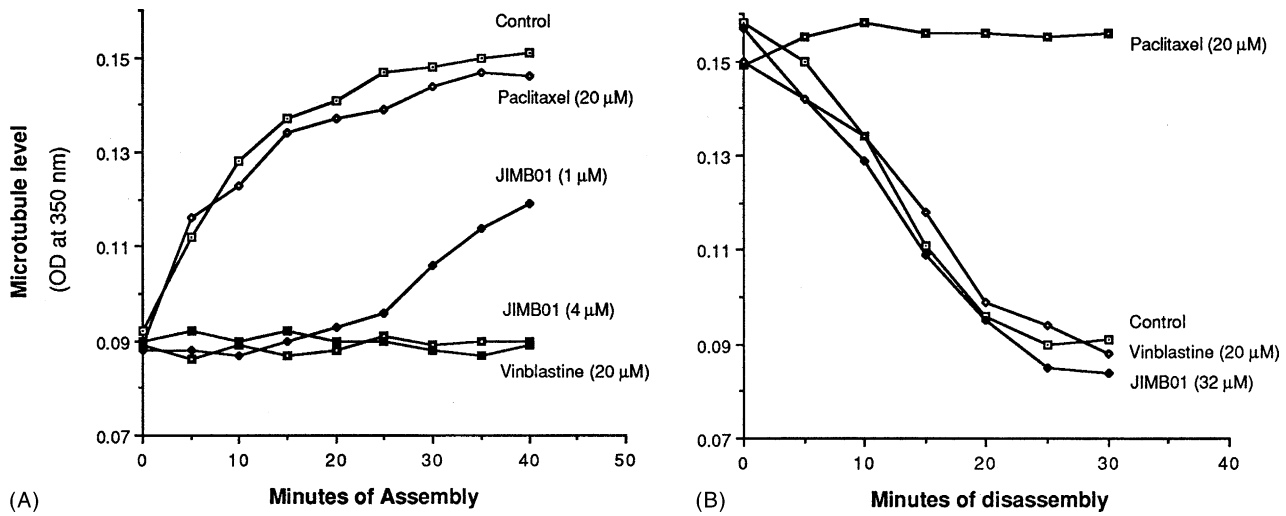

Fig. 7. Effect of JIMB01 on the microtubule assembly–disassembly cycle. (A) Free tubulins in reaction buffer were incubated with GTP and Mg^{2+} at RT for assembly in the absence or presence of JIMB01 (1 or 4 μM), or paclitaxel (20 μM), or vinblastine (20 μM). (B) Assembled microtubules were incubated in ice for disassembly in the absence or presence of JIMB01 (32 μM), or paclitaxel, or vinblastine at the concentrations mentioned above. Microtubule assembly or disassembly was determined every 5 min by O.D. at 350 nm. The experiment was performed three times.

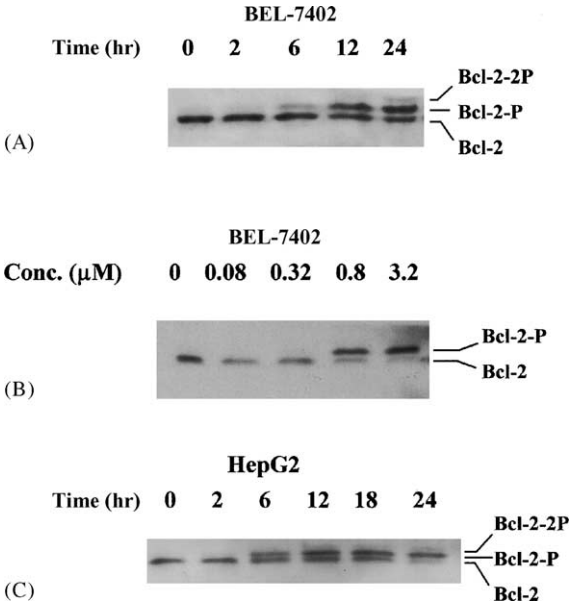

Fig. 8. JIMB01-induced phosphorylation of Bcl-2 in hepatocarcinoma cell lines. (A) BEL-7402 cells were treated with JIMB01 (3.2 μM) for 0, 2, 6, 12, and 24 hr. (B) BEL-7402 cells were incubated with JIMB01 at concentrations of 0, 0.08, 0.32, 0.8, and 3.2 μM for 24 hr. (C) HepG2 cells were treated with JIMB01 (3.2 μM) for 0, 2, 6, 12, 18, and 24 hr. Phosphorylated Bcl-2 is labeled as Bcl-2-P for one phosphorylation and Bcl-2-2P for two phosphorylations.

3.6. Bcl-2 phosphorylation

Considering that all microtubule-active agents inactivate Bcl-2 through its phosphorylation [21,22], the change in the phosphorylation state of Bcl-2 in response to JIMB01 was examined. The earliest Bcl-2 phosphorylation, characterized as a slower-migrating band, was observed in BEL-7402 and HepG2 cells 6 hr after treatment (Fig. 8A and C). Treatment of BEL-7402 cells with JIMB01 at 0.8 μM or above clearly induced Bcl-2 phosphorylation (Fig. 8B). Leukemic cells treated with JIMB01 exhibited similar results (not shown).

3.7. Inhibition of the growth of human liver cancer in nude mice

As shown in Fig. 9, when the average BEL-7402 tumor volume in the solvent-treated controls was above 2000 mm^3 on day 35 post-implantation, the group treated with 15 mg/kg (i.p.) JIMB01 had a tumor size one-third that of the solvent controls, and the tumor size of the 10 mg/kg group was less than one-half that of the controls. Apparently, BEL-7402 tumor proliferation *in vivo* was inhibited significantly by the compound (JIMB01 15 mg/kg group vs untreated controls, $P = 0.01$). Furthermore, vincristine was used as the control of a known microtubule-active agent in the study. JIMB01 at 15 mg/kg (causing 66% inhibition in tumor volume) appeared to have a greater inhibitory effect on tumor growth than vincristine (causing 43% inhibition), although it was not statistically significant ($P = 0.08$). The dose of vincristine used in this experiment was 0.3 mg/kg (i.p.), close to its toxic dose [23]. Regression of the preformed tumor in mice treated with JIMB01 was not evidenced.

Visible toxicity of JIMB01 was not observed in the course of the treatment. The average body weight of the mice in the 15 mg/kg treated group was 19 ± 1.4 (g) before treatment (day 1), and 20 ± 2.1 (g) when the experiment

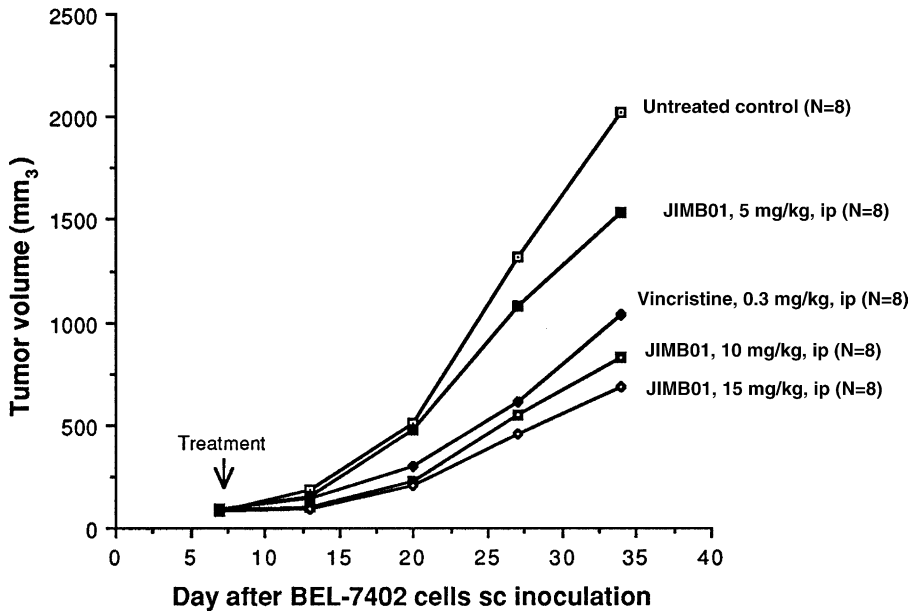

Fig. 9. Inhibition of a human hepatocarcinoma (BEL-7402) by JIMB01 in nude mice. BEL-7402 tumor tissue (4 mm^3) was transplanted s.c. into the mice. Seven days later, mice with a visible tumor mass were randomly distributed into cages with 8 mice per group. Treatment (JIMB01 at 5, 10, or 15 mg/kg, i.p.; vincristine at 0.3 mg/kg, i.p.) was started on day 8, and the regimens were continued with another six injections at 4-day intervals. Each point represents the mean of the tumor volume (N = 8). Error bars are not shown to preserve the clarity.

was terminated (day 35). The LD_{50} of JIMB01 (i.p.) in nude mice was 37 mg/kg.

4. Discussion

Microtubules are dynamic assemblies of tubulin units and essential for distributing chromosomes equally into two daughter cells in cell division. The tubulin dynamics increase up to 100-fold in mitosis as compared to interphase [24], and the half-time of exchanging spindle tubulin with tubulin in the free tubulin pool is about 10 sec [25]. This high-speed dynamic is sensitive to many agents targeting tubulin or the intact microtubule, such as vinca alkaloids, paclitaxel, and others [24,26–28]. The primary action of these therapeutic agents is to bind to sites on tubulin or microtubules, causing an alteration of the tubulin dynamics and, subsequently, a block in mitosis [28]. Disruption of microtubules by any of the microtubule-active agents results in a phosphorylated form of Bcl-2 [22,29,30], which is without activity [30] and fails to bind to Bax [30]. This process leads Bcl-2 positive tumor cells to apoptosis [21]. The cell death pathway mentioned above appears to be applicable to JIMB01. The antiproliferative effect of JIMB01 was clearly mediated by apoptosis, as demonstrated morphologically by the fragmentation of condensed chromatin and electrophoretically by the characteristic DNA degradation ladder in tumor cells exposed to JIMB01. The apoptosis caused by JIMB01 was associated with a profound accumulation of M-phase cells, increased kinase activity of cyclin B1 (which is the operating enzyme responsible for mitosis [31]), and Bcl-2 inactivation (phosphorylation). The primary target of JIMB01 was the assembly of microtubules from tubulin in dividing cells. The mode of action of JIMB01 was similar to that of vinca alkaloids, and differed from that of paclitaxel.

P-gp-expressing multidrug-resistant tumor cells have a diminished susceptibility to the induction of apoptosis by microtubule-active agents, most of which are considered substrates of P-gp [32,33]. This is one of the major drawbacks of these agents in clinical chemotherapy. The fact that Daudi/MDR cells, which express P-gp and are resistant to vincristine and doxorubicin [34], were as susceptible to JIMB01 as Daudi/wt cells shows clearly that JIMB01 can bypass the mechanism of chemoresistance from P-gp. In addition, the fact that HL60 leukemia cells, which are p53 null, were as sensitive to the compound as p53-expressing CEM leukemia cells suggests an apoptotic mechanism for JIMB01 action that is independent of p53.

We focused our attention in the present study on the effect of JIMB01 on human hepatocarcinoma cells for two reasons. First, human hepatocellular carcinoma (HCC) is one of the most prevalent malignancies in China; and second, microtubule-active agents are commonly selected for chemotherapy in the clinic for patients with hepatocarcinoma [35,36]. Three hepatocarcinoma cell lines originating from Chinese patients and one hepatoma line of non-Chinese origin were tested *in vitro*. These human hepatocarcinoma cell lines showed a similar susceptibility to the compound, with the exception of the Chinese line SMMC-7721, of which information of its phenotype or genotype has not been documented. Since JIMB01 was not a substrate of P-gp, inactivated the Bcl-2 anti-apoptotic function, and induced apoptosis independently of p53, possible explanations for the resistance of SMMC-7721 are: (a) mutations on the molecules (e.g. Bcl-2) involved in the apoptotic pathway employed by JIMB01; (b) a slower uptake and/or generation time; and (c) a particular phenotype of tubulin (for instance, isoforms of β-tubulin) that is not favorable for the binding of JIMB01.

For the efficacy of JIMB01 *in vivo*, the inhibitory effect on the growth of BEL-7402 human hepatocarcinoma was substantial and dose-dependent. Treatment with higher doses was more effective than with lower doses, compatible with the theory that high-dose chemotherapy may be a potentially curative treatment in some tumors [37]. Also important, with respect to body weight, was the fact that JIMB01 was well tolerated in the tumor-bearing mice treated with 15 mg/kg (i.p.) for seven injections. No toxic response was observed during the treatment. Compared with the known microtubule agent vincristine (MW 923), JIMB01 has a much smaller molecular weight and can be readily synthesized in a few chemical reaction steps. JIMB01 demonstrated an inhibitory effect on the growth of human BEL-7402 hepatocarcinomas in nude mice that was comparable to that of vincristine. The efficient activity of JIMB01 against human hepatocarcinomas *in vivo* was probably due to: (a) its favorable bioavailability in the blood circulation and handling by the liver; and (b) its induction of an apoptotic pathway that is independent of the one involved in MDR in tumor cells.

Considering that JIMB01 had a specific molecular target, the microtubule, induced apoptosis in tumor cells, bypassed the MDR mechanism, and showed promising activity against a human hepatocarcinoma in nude mice, we recommend that JIMB01 be investigated further as a new cancericidal tubulin ligand.

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