Cancer Chemother Pharmacol (1999) 44: 444–452

ORIGINAL ARTICLE

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Apoptotic cell death induced by baccatin III, a precursor of paclitaxel, may occur without G_2/M arrest

Received: 13 December 1998 / Accepted: 10 May 1999

Abstract *Purpose*: Paclitaxel has been demonstrated to possess significant cell-killing activity in a variety of tumor cells by induction of apoptosis, but the mechanism by which paclitaxel leads to cell death and its relationship with mitotic arrest is not entirely clear. In this study, baccatin III, a synthetic precursor of paclitaxel, was used to analyze whether paclitaxel-induced apoptosis can be a separate event from microtubule bundling and G_2/M arrest. *Methods*: Several different methods including DNA fragmentation, flow cytometric analyses, TdT-mediated dUTP nick end labeling (TUNEL) and time-lapse video microscopy were used to analyze apoptotic cell death induced by baccatin III and its possible correlation with cell cycle distribution. *Results*: Our results demonstrated that baccatin III could also cause apoptotic cell death in both BCap37 (a human breast cancer cell line) and KB cells (derived from human epidermoid carcinoma), but had less effect on microtubule bundling and G_2/M arrest. Furthermore, we demonstrated that most apoptotic events induced by baccatin III were not coupled with G_2/M arrest. Instead, these apoptotic events occurred predominantly in the cells in other phases of the cell cycle. *Conclusion*: Baccatin III, which contains the core taxane ring, is the fundamental piece of paclitaxel structure. The finding of baccatin III-induced apoptosis independent of cell cycle arrest, on the one hand, implies that the core taxane ring may play a critical role in inducing cell death and, on the other hand, suggests that paclitaxel might induce apoptosis from other phases of the cell cycle by a similar mechanism.

Key words Baccatin III · Paclitaxel · Apoptosis · Mitotic arrest · bcl-2

Abbreviations: *PI* propidium iodide; *SDS* sodium dodecylsulfate; *TdT* terminal deoxynucleotidyl transferase

Introduction

Paclitaxel (Taxol®) is a multifunctionalized diterpenoid first isolated in 1971 from the inner bark of the western yew tree (*Taxus brevifolia*) [1]. This naturally occurring agent has been demonstrated to possess broad activity against human solid tumors, particularly in drug-refractory ovarian cancer and metastatic breast cancer [2–5]. However, the exact mechanism of paclitaxel's cytotoxicity against tumor cells is not entirely clear. Previous studies have demonstrated that paclitaxel is a unique antimicrotubule agent [6]. Unlike classical antimicrotubule agents such as vinblastine and colchicine that induce microtubule disassembly and paracrystal formation, paclitaxel induces tubulin polymerization and promotes the formation of unusually stable microtubules [7]. This inhibits the normal dynamic equilibrium of microtubules required for cell division and proliferation [8, 9]. Therefore, it has generally been believed that paclitaxel's antitumor effects result from interference with the normal function of microtubules and cell cycle arrest in late G_2/M phases via disruption of mitotic spindles [10].

In recent years, paclitaxel has been demonstrated to induce internucleosomal DNA fragmentation and morphological changes characteristic of apoptosis in a variety of solid tumor cells [11, 12]. These findings indicate that paclitaxel, in addition to its antimicrotubule and cell cycle arrest activities, possesses significant cell-killing activity by inducing apoptosis. In many solid tumor cells, paclitaxel can cause both mitotic arrest and

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apoptotic cell death, but it is unclear whether paclitaxel-induced cell death is a secondary event resulting from mitotic arrest or represents a novel mechanism of action of paclitaxel against tumor cells.

Morphologically, a sustained block of mitosis seems to be required for paclitaxel-induced apoptosis because, by time-lapse video microscopy, most apoptotic events have been observed to occur only in cells showing prior mitotic arrest [12]. However, this observation does not prove that apoptosis induced by paclitaxel is a secondary event resulting only from mitotic arrest. In fact, several lines of evidence suggest that paclitaxel-induced apoptosis might take place via an independent pathway (reviewed in reference 13). For example, it has been reported that low concentrations of paclitaxel (≥10 n*M*) can result in abnormal mitotic exit and consequent apoptosis without a G_2/M arrest [14]. In addition, through investigation of paclitaxel cytotoxicity in HeLa 53 cells, Donaldson et al. have recently suggested that mitotic block might not be a sufficient signal for apoptotic cell death. Instead, their results indicate that paclitaxel-induced apoptosis may occur by way of a phosphoregulatory pathway, possibly involving $p34^{cdc2}$ kinase [15]. Also, recent studies have shown that paclitaxel can induce oncoprotein bcl-2 phosphorylation [16]. This posttranslational modification of bcl-2 is believed to decrease bcl-2's ability to block apoptosis [17]. Based on these results and the fact that paclitaxel-induced mitotic arrest occurs fairly rapidly, it is highly possible that paclitaxel-induced apoptosis may be via a distinct signaling pathway that occurs coincidentally in mitotically arrested cells.

Unfortunately, paclitaxel has such unmistakable effects on microtubules and mitotic arrest, it is difficult to observe any underlying mechanisms involved in paclitaxel-induced apoptosis. Baccatin III, which contains the core taxane ring, is the synthetic precursor of paclitaxel (Fig. 1). Previous studies have shown that it has less effect on microtubules [18, 19]. In mammalian cells, the bioactivity of baccatin III as an inhibitor of tubulin disassembly has been found to be about 50-fold less than that of paclitaxel [19]. Considering that baccatin III is the backbone in the structure of paclitaxel and has low affinity for microtubules, we decided to compare this compound with paclitaxel with regard to their cytotoxic effects on microtubule bundling, mitotic arrest and induction of apoptosis in an attempt to evaluate the possibility that paclitaxel-induced apoptois occurs independently of mitotic arrest. Two human tumor cell lines, BCap37 (a breast cancer cell line) and KB (derived from a human epidermoid carcinoma), were used for this study because they have shown sensitivities to paclitaxel in both mitotic arrest and apoptosis [12, 20]. The results presented here show that baccatin III, at suitable concentrations, can induce tumor cell apoptosis without G_2/M arrest. This finding implies that baccatin III and possibly paclitaxel may induce apoptotic cell death via a signaling pathway that is independent of cell cycle arrest.

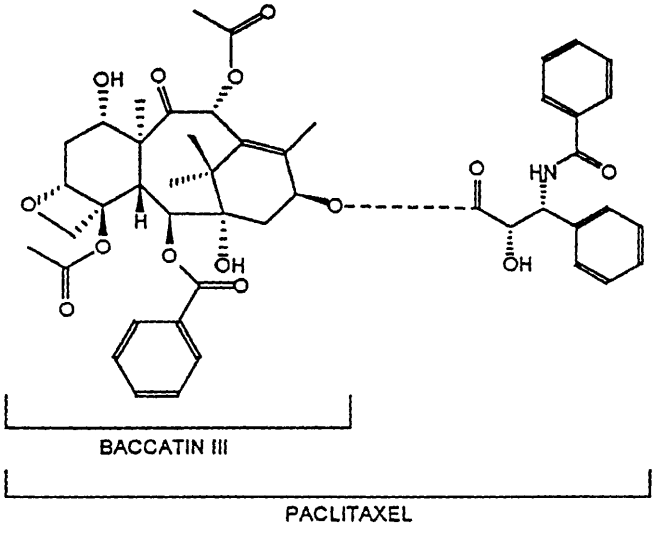

Fig. 1 Baccatin III is a fundamental piece of paclitaxel's structure

Materials and methods

Drugs and cell culture

Paclitaxel and baccatin III purchased from Sigma Chemical Co. (St. Louis, Mo.) were dissolved in 100% dimethyl sulfoxide (DMSO) to make a stock solution of 1 m*M*, which was then diluted in culture medium to obtain the desired concentration. BCap37 human breast cancer [12] and KB human epidermoid carcinoma (American Type Culture Collection, Rockville, Md.) cell lines were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum plus penicillin, streptomycin, and Fungizone®. As previously described, paclitaxel or baccatin III was added when the cells had reached approximately 60–70% confluency [12].

Determination of internucleosomal DNA cleavage

Internucleosomal DNA fragmentation was assayed by a modification of previously described methods [12, 20]. After treatment of cells with various concentrations of baccatin III or paclitaxel, cells were harvested, counted and washed with phosphate-buffered saline (PBS) at 4 °C. Cells were suspended in lysis solution containing 5 m*M* Tris-HCl, 20 m*M* EDTA, and 5% (v/v) Triton X-100 for 30 min on ice. The remaining steps for DNA fragmentation were performed exactly as previously described [12]. DNA samples were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.2 μg/ml ethidium bromide, and visualized under UV illumination.

Flow cytometry analysis

Cell sample preparation and propidium iodide (PI) staining were performed according to the method described by Nicoletti et al. [21]. Briefly, cells were treated with baccatin III [1–10 μM] or paclitaxel (100 n*M*) for 24 and 48 h. Cells were then harvested by trypsinization and washed twice with PBS. Cells were fixed in 1% formaldehyde in PBS on ice, then dehydrated in 70% ethanol in PBS. Approximately 1 h before flow cytometry analysis, RNase A (1 mg/ml) and PI (10 μg/ml) were added to each sample. Samples were allowed to incubate in complete darkness at room temperature for 30 min. Cell cycle distribution was determined using a Coulter Epics V instrument (Coulter Corporation, USA) with an argon laser set to excite at 488 nm. The results were analyzed using Elite 4.0 software (Phoenix Flow System, San Diego, Calif.).

Morphological examination through cytospin preparation

Cells treated with different concentrations of baccatin III were harvested by trypsinization at the times indicated and washed twice with PBS. Cell numbers were determined with a hemocytometer and approximately $0.5–1 \times 10^5$ cells were plated onto microscope slides using a Cytospin 3® cell preparation system (Shandon, Pittsburgh, Pa.). Slides were air dried and fixed in acetone prior to Wright-Giemsa staining. The number of mitotic cells was determined using bright field microscopy.

Analysis of microtubular structure by immunofluorescence

BCap37 cells cultured in 35-mm dishes were treated with paclitaxel or baccatin III. After 24 h, the dishes were washed three times with PBS, and treated and fixed with 3.7% formaldehyde in PBS for 30 min. The cells were then incubated in 0.1% saponin and 4 mg/ml normal goat globulin with a monoclonal rat antitubulin antibody (Accurate Chemical, Westbury, N.Y.) for 30 min at room temperature. After washing with PBS, the cells were incubated with affinity-purified rhodamine-conjugated goat antirat IgG (Jackcon Immuno Research, West Grove, Pa.). The dishes were viewed and photographed with a Zeiss Axioplan epifluoresence microscope equipped with rhodamine filter set.

Time-lapse video phase-contrast microscopy

Cells treated with baccatin III or paclitaxel in 100-mm flasks were placed on the stage of a warmed inverted microscope and examined using phase-contrast microscopy as previously described [22]. This microscope is equipped with a heated recirculation device that maintains stage temperature at 37 ± 0.5 °C. In addition, this system is supplied with a constant flow of 95% air/5% CO_2. Time-lapse video recordings were prepared at a 1:720 time-lapse ratio over 72 h.

Detection of DNA fragmentation and cell cycle distribution using bivariate flow cytometry

TdT-mediated dUTP nick end labeling (TUNEL) and PI staining of cells followed the method of Juan and Darzynkiewicz [23]. Briefly, $10^6–2 \times 10^6$ cells treated with baccatin III or paclitaxel were fixed in ice-cold 1% formaldehyde in PBS for 15 min then dehydrated in ice-cold 70% ethanol. The samples were then centrifuged (300 *g*, 5 min) and washed with PBS to remove the ethanol. The cell pellet was suspended in 50 µl buffer containing 1× reaction buffer (Promega, Madison, Wis.), 80 µ*M* BrdUTP, and 12.5 U terminal deoxynucleotidyl transferase (TdT; Promega, Madison, Wis.). Cells were incubated in this solution for 40 min at 37 °C then rinsed in buffer containing 0.5% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS. The cell pellet was resuspended in anti-BrdU-fluorescein mouse monoclonal antibody (clone BMC 9318; Boehringer Mannheim, Indianapolis, Ind.) (5 µg/ml in PBS with 1% BSA and 0.3% Triton X-100) and incubated at room temperature for 1 h. The cells were then washed with PBS and resuspended in PBS containing 0.1 mg/ml RNase A and 5 µg/ml PI. The cells were allowed to stain with PI for at least 30 min before analysis by flow cytometry. Negative controls were made by omitting TdT. Cell cycle distribution was determined using a Coulter Epics V instrument (Coulter Corporation, USA). PI and fluorescein fluorescence signals were collected using linear and logarithmic scales, respectively. TUNEL-positive events were defined by setting a gating window to exclude greater than 99% of negative control events. The results were analyzed using Elite 4.0 software (Phoenix Flow System, San Diego, Calif.).

Western hybridization

Cells treated with baccatin III or paclitaxel were harvested by trypsinization after 24 h exposure. Cellular proteins were extracted with a buffer containing 1.0% Triton X-100 in PBS containing 0.5 m*M* EDTA and the recommended concentration of protease inhibitor cocktail (Boehringer Mannheim). The protein samples were loaded onto a 12% SDS polyacrylamide gel at equal protein concentrations. After electrophoresis, samples were transferred to a nitrocellulose membrane according to the BioRad protocol. Bcl2 was localized using antibody no. 124 (DAKO) at 1:1000 dilution (~0.3 µg/ml) in 3% BSA-PBS-T (PBS containing 0.5% Tween 20), and a secondary antibody, goat antimouse IgG, conjugated to horseradish peroxidase at a concentration of 0.1 µg/ml in 3% BSA-PBS-T (Jackson ImmunoResearch). The reactive bcl2 band was identified using a chemiluminescent substrate to horseradish peroxidase (Amersham).

Results

Baccatin III induces DNA fragmentation in both BCap37 and KB cells

An important feature of apoptotic cell death is the fragmentation of genomic DNA into integer multiples of 180-bp units producing a characteristic ladder on agarose gel electrophoresis [24]. First, we used the DNA fragmentation assay to determine whether baccatin III can also cause apoptosis. Figure 2 shows the DNA fragmentation of BCap37 and KB cells treated with different concentrations of baccatin III and paclitaxel. As previously reported [12, 24], characteristic DNA fragmentation ladders were observed in both BCap37 and KB cells after treatment of cells with 100 n*M* pac-

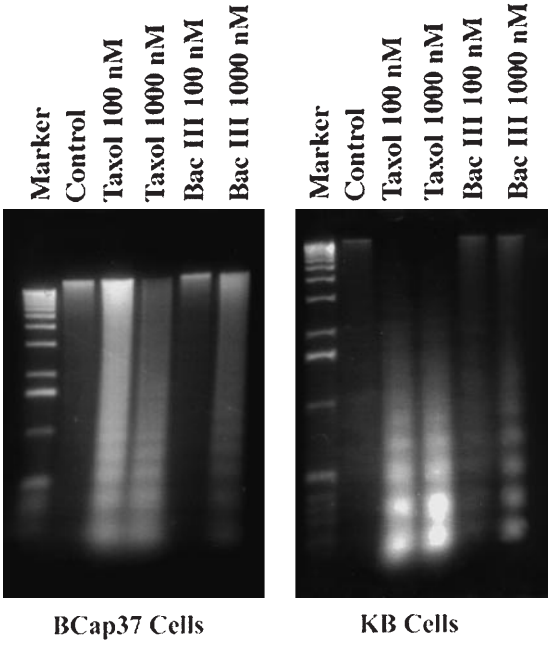

Fig. 2 Baccatin III-induced internucleosomal DNA fragmentation in BCap37 and KB cells. Cells were treated with paclitaxel or baccatin III (100 or 1000 n*M*). The control group contained 0.1% DMSO (v/v). Following a 72-h drug exposure, cells were harvested and fragmented DNA was extracted as described in Materials and methods. Fragmented DNA was analyzed by electrophoresis in a 1.2% agarose slab gel with 0.2 µg/ml ethidium bromide

litaxel for 72 h (Fig. 2), but baccatin III at this concentration (100 n*M*) had little effect in inducing DNA fragmentation, although a faint ladder was visible in KB cells (Fig. 2, lane 5). However, the characteristic ladders associated with DNA fragmentation were observed for both BCap37 and KB cells when they were treated with 1 μ*M* baccatin III for 72 h (Fig. 2, lane 6). These results indicate that baccatin III can also cause apoptotic cell death, but requires a higher concentration than paclitaxel.

Table 1 Comparison of baccatin III and paclitaxel in inducing mitotic arrest. Cytospin slides were stained with Giemsa. From each slide, 300 cells were counted and only those with typical morphological features of condensed chromosomes were recorded as mitotically arrested G_2/M phase cells. Values are the percentage of cells (mean ± s.e.m) in G_2/M phase based on three separate experiments

Drug exposure	Bcap37 cells		KB cells	
	24 h	48 h	24 h	48 h
Control	6 ± 2	5 ± 3	5 ± 2	6 ± 3
1 μ*M* baccatin III	15 ± 4	21 ± 5	11 ± 3	19 ± 6
10 μ*M* baccatin III	47 ± 6	38 ± 10	50 ± 6	35 ± 7
100 n*M* paclitaxel	63 ± 7	41 ± 8	52 ± 7	39 ± 11

Baccatin III has a lower capacity for inducing G_2/M arrest

We next examined the effect of baccatin III on mitotic arrest. By flow cytometric analyses, we observed that baccatin III (1 μ*M*), unlike paclitaxel, only caused marginal increases (about 10%) in the proportion of cells in G_2/M phase of the cell cycle in both BCap37 and KB cells (Fig. 3), although significant DNA fragmentaion was observed for this concentration of baccatin III. Further, we used the cytospin method to determine the percentage of mitotically arrested cells in these tumor cell lines treated with baccatin III for 24 and 48 h. The results summarized in Table 1 indicate that the percentage of mitotically arrested cells (with clearly condensed chromosomes) in both tumor lines treated with 1 μ*M* baccatin III for 24 h was only 15% and 11%, respectively. In contrast, 63% and 52% of the cells were arrested in G_2/M phase of the cell cycle when BCap37 and KB cells were treated with 100 n*M* paclitaxel for 24 h. By 48 h, the percentage of cells in G_2/M phase was slightly increased in baccatin III groups, but the number of typical G_2/M cells in the paciltaxel group was decreased because a large number of cells arrested in G_2/M had undergone apoptosis. However, when these tumor cells were treated with 10 μ*M* baccatin III, about 50% of the cells were arrested in G_2/M phase in both KB and BCap37 cells, which is comparable to the arrest caused by 100 n*M* paclitaxel. These results indicate that higher concentrations of baccatin III (over 10 μ*M*) were still able to achieve G_2/M arrest similar to paclitaxel (100 n*M*) in both BCap37 and KB cell lines.

Baccatin III has little effect on microtubule bundling

A key feature of paclitaxel-treated cells is the formation of abnormally stable microtubule bundles [8, 9]. By immunofluorescence, we were able to see that paclitaxel (100 n*M*) produced significant rounding of tumor cells and "hoop-like" bundles of microtubules (Fig. 4) [20]. However, 1 μ*M* baccatin III seemed to have very little, if any, effect on bundling of microtubules. As presented in Fig. 4, the BCap37 cells treated with 1 μ*M* baccatin III showed a centriole-related radiating array of microtubules in a delicate, fine network, which was not significantly different from the normal microtubule pattern. However, increasing the concentration of baccatin III to 10 μ*M* resulted in the appearance of readily distinguishable parallel microtubule bundles in BCap37 cells (see Fig. 4). These results were in parallel with the observation of baccatin III's effect on mitotic arrest, suggesting that baccatin III may affect microtubule

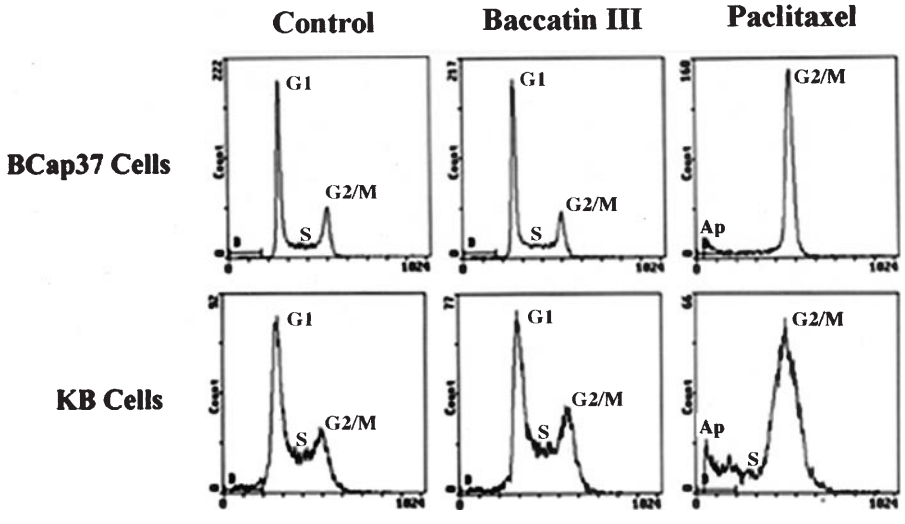

Fig. 3 Flow cytometric analysis of BCap37 and KB cell cycle distribution. Cells were treated with 1 μ*M* baccatin III or 100 n*M* paclitaxel. Controls contained 0.1% DMSO (v/v). Following a 24-h incubation, cells were harvested, stained for DNA with PI and flow cytometry was performed as described in Materials and methods. For each sample 10^5 events (cells) were recorded. The peaks corresponding to G_0/G_1, S, and G_2/M phases of the cell cycle are indicated

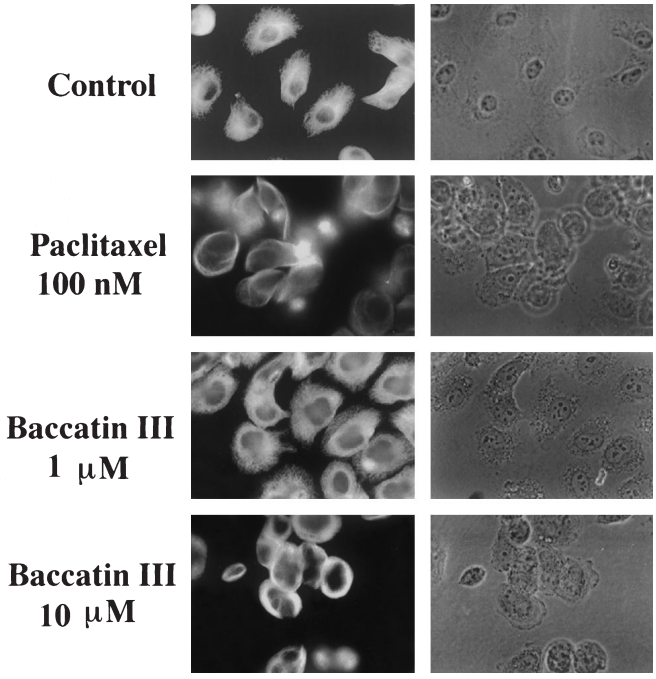

Fig. 4 Examination of the effects of baccatin III on microtubule structure. BCap37 cells seeded in 35-mm dishes were treated with baccatin III or paclitaxel for 24 h. The dishes were washed three times and fixed with formaldehyde, incubated with saponin and then with monoclonal rat antitubulin antibody. After washing with PBS, the cells were incubated with rhodamine-conjugated goat antirat IgG. Photographs were made using an epifluorescence microscope equipped with a rhodamine filter set and phase contrast optics

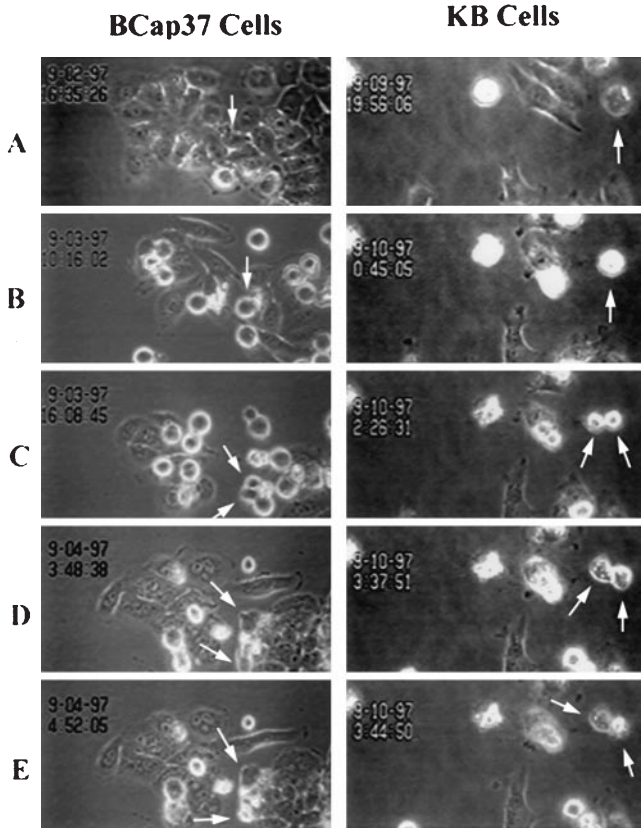

Fig. 5A–E Time-lapse video microscopy of BCap37 and KB cells. Cells treated with 1 μ*M* baccatin III were visualized using phase-contrast microscopy. Time-lapse video recordings were prepared at a 1:720 time-lapse ratio over 72 h. Still images were obtained from video tape from which cells can be seen to pass through mitosis and enter subsequent phases of the cell cycle. These cells then undergo apoptosis without reentering G_2/M arrest (*arrowed*). **A** photographed at time 0, **B** cell entering mitosis, **C** cell division, **D** reattachment, **E** apoptosis

bundling by the same mechanism as paclitaxel although it has a less-potent interaction with microtubules.

Baccatin III-induced apoptosis occurs in other phases of the cell cycle

Since 1 μ*M* baccatin III has only limited effects on mitotic arrest and microtubule bundling, we expected that at least some of the apoptotic events might occur in other phases of the cell cycle. Phase-contrast video microscopy showed that only a small percentage of cells died from mitotic arrest when BCap37 or KB tumor cells were treated with 1 μ*M* baccatin III, while the majority of apoptotic events occurred during other phases of the cell cycle (Fig. 5). The video recordings showed that many tumor cells, after initial treatment with baccatin III (1 μ*M*), passed through mitosis and entered subsequent phases of the cell cycle (arrowhead in Fig. 5B to D) and then underwent apoptosis without reentering G_2/M phase of the cell cycle (Fig. 5E). Figure 6 summarizes these results based on three experiments. It is apparent that approximately 40% of baccatin III-treated BCap37 cells underwent apoptosis from phases other than G_2/M while a similar proportion (~49%) of KB cells underwent apoptosis from other phases of the cell cycle during 72 h of videomonitoring. A smaller percentage of baccatin III-treated BCap37 and KB cells (7% and 24%, respectively) died following G_2/M arrest. Thus, these results provide direct evidence that apoptotic cell death caused by baccatin III can occur in other phases of the cell cycle.

In addition, the cell cycle distribution of apoptotic cells was analyzed by the TUNEL method in combination with PI staining and flow cytometry. By labeling cells by both TUNEL and PI staining made it possible to quantify the incorporation of BrdUTP relative to DNA content. As shown in Fig. 7, 10.6% and 33.4% of cells treated with 1 μ*M* baccatin III were TUNEL-positive after 24 and 48 h, respectively. These cells appeared to be evenly distributed throughout the cell cycle, suggesting that DNA fragmentation and apoptosis in these cells was independent of cell cycle. In comparison, 24.6% and 97.4% of cells treated with 100 n*M* paclitaxel were TUNEL-positive after 24 and 48 h, respectively. Since the majority of these cells were arrested in G_2/M of the cell cycle, most apoptotic events obviously occurred

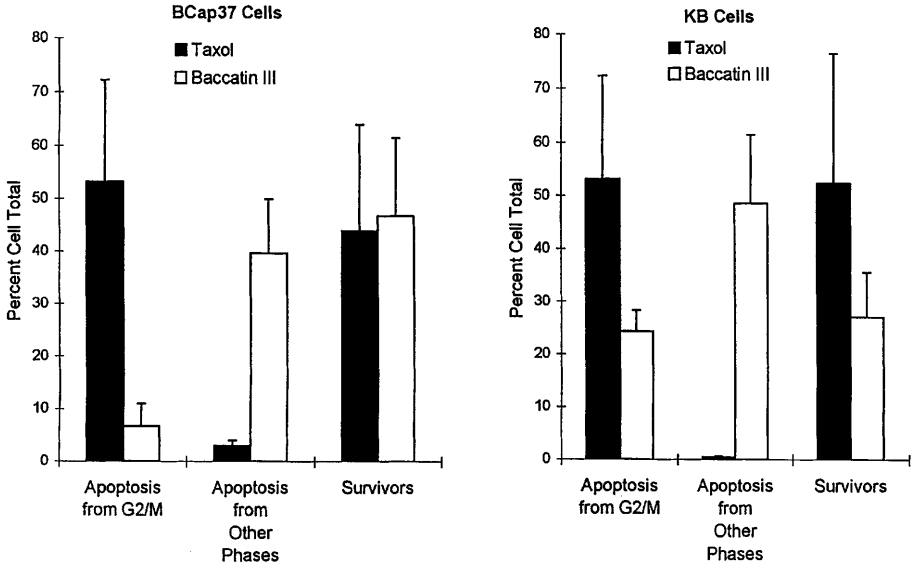

Fig. 6 Baccatin III-induced apoptotic cell death from G_2/M or other phases of the cell cycle. BCap37 or KB cells were seeded in 25-mm flasks which were placed on the stage of a warmed inverted microscope and examined using phase-contrast microscopy as described in Materials and methods. Individual cells in selected video fields were identified as they were rounded into mitotic arrest. Those individual cells were then followed to observe their fate; they either showed the morphological features of apoptosis (blebbing and cell shrinkage) or passed through mitosis. Apoptotic events occurring from other phases of the cell cycle were also counted. Data in the bar graph are the means ± SD of three experiments. An average of 53 ± 15 BCap37 cells or 41 ± 7 KB cells per field of view were observed in the three experiments

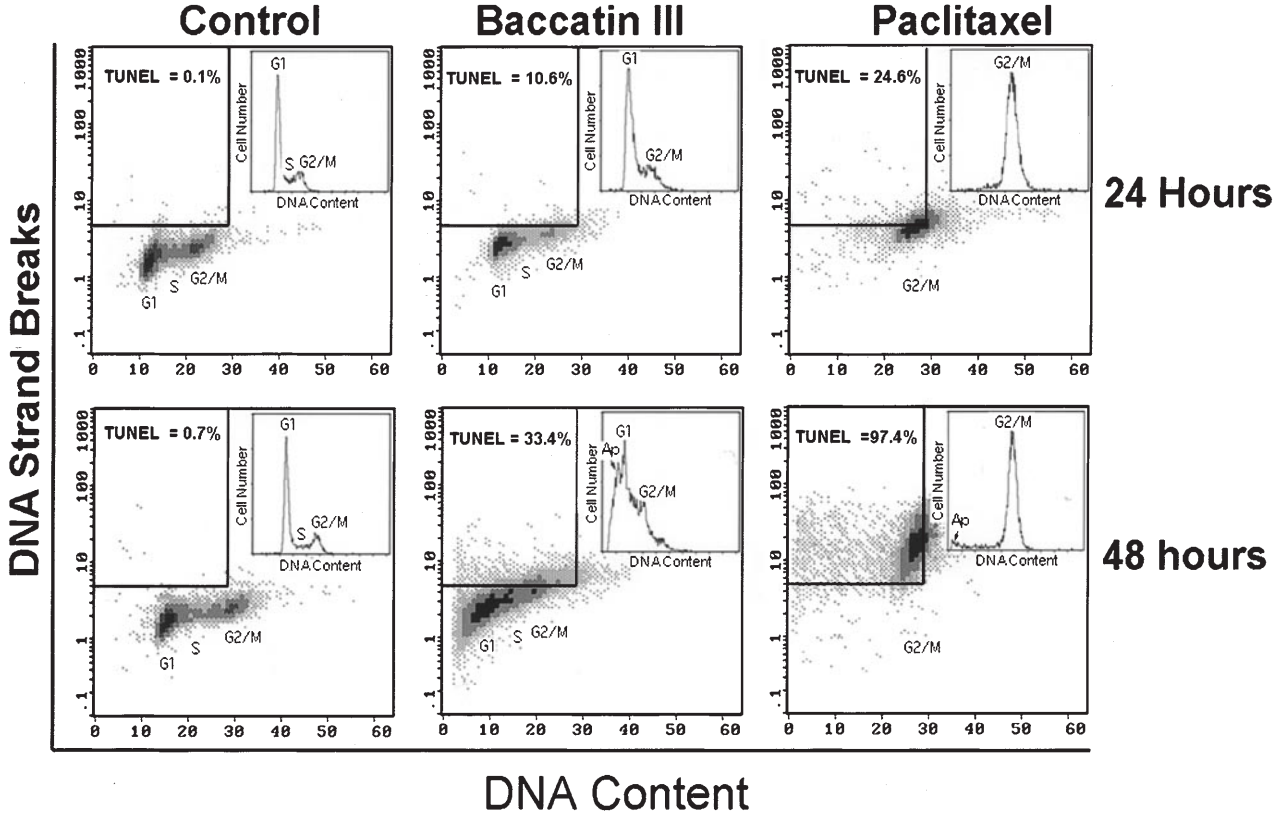

Fig. 7 Cell cycle distribution of apoptotic cells analyzed by differential labeling of DNA strand breaks and fluorescence measurement by flow cytometry. Bivariate distributions (scattergrams) represent the intensity of DNA strand break labeling versus cellular DNA content. BCap37 cells were treated with baccatin III (1 μ*M*) or paclitaxel (100 n*M*) for 24 or 48 h. DNA strand breaks were indirectly labeled with BrdUTP which was then labeled with an anti-BrdU-fluorescein mAb. DNA histograms (*insets*) represent all cells. For each sample 10^5 events (cells) were recorded. "TUNEL" in the upper lefthand corner of each panel indicates the percentage of TUNEL positive cells in the gated area (*rectangle*)

following the mitotic arrest. It is also apparent, however, that a smaller percentage of these cells underwent DNA fragmentation in other phases of the cell cycle (Fig. 7).

Baccatin III also causes hyperphosphorylation of Bcl-2

We have previously demonstrated that paclitaxel can cause bcl-2 hyperphosphorylation in both BCap37 and

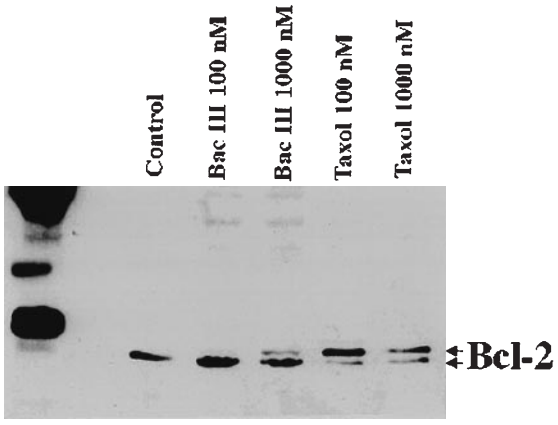

Fig. 8 Evaluation of bcl-2 protein content by Western analysis. Cellular proteins were extracted from BCap37 cells treated with baccatin III or paclitaxel for 24 h. Cellular protein (100 μg/lane) was separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blotted with mouse anti-bcl-2 antibody and analyzed as described in Materials and methods

KB cells [25, 26]. To determine whether baccatin III produces similar effects on bcl-2 phosphorylation, Western blots of bcl-2 protein extracted from baccatin III-treated tumor cells were performed. The results shown in Fig. 8 indicate that baccatin III (1 μ*M*) was also able to induce a detectable increase in hyperphosphorylated bcl-2. This result correlates well with the concentration of baccatin III necessary to cause DNA fragmentation and suggests that baccatin III and paclitaxel may induce apoptotic cell death by similar mechanisms.

Discussion

Although paclitaxel has been demonstrated to cause both mitotic arrest and apoptotic cell death in a variety of tumor cells, the possible correlation between these two events remains unclear. One major reason is that paclitaxel's cytotoxic effects on microtubules and cell cycle arrest are so dominant that most of the apoptotic events are observed only in cells previously arrested in the G_2/M phase of the cell cycle [12]. Thus, to determine if paclitaxel-induced apoptosis can occur independently of mitotic arrest, one feasible approach was to find a paclitaxel-related compound with similar biological activity but with less effect on microtubules. Therefore, the synthetic precursor of paclitaxel, baccatin III, was used in this study to address the possibility that there may be an apoptotic pathway independent of mitotic arrest. The results presented here establish that baccatin III, at suitable concentrations, can selectively induce apoptotic cell death with minor effects on microtubular structure or cell cycle arrest. More importantly, this study has provided direct visual evidence that baccatin III can induce apoptotic cell death in cells that are not mitotically arrested.

It has been well established that paclitaxel causes microtubule bundling and subsequent cell cycle arrest in G_2/M phase [8–10]. Photoaffinity studies have indicated that paclitaxel interacts specifically with the β subunit of tubulin, and there appears to be a 1:1 stoichiometry in the interaction of paclitaxel with tubulin [27, 28]. What is not clear, however, is the possible correlation between mitotic arrest and apoptotic cell death. In recent years, we and others have obtained several lines of evidence that paclitaxel may induce apoptosis via a separate pathway from mitotic arrest. For example, our recent experiments have demonstrated that apoptotic cell death in BCap37 cells is triggered as long as the cells have been exposed to 100 n*M* paclitaxel for only 1 h [12]. This phenomenon indicates that paclitaxel-induced apoptosis is an irreversible process, which contrasts with the reversible manner of paclitaxel binding to microtubules [8]. In addition, paclitaxel demonstrates other properties that may allow alternate cytotoxic mechanisms. These include the ability to activate lipopolysaccharide-induced genes [29, 30], induction of bcl-2 phosphorylation [16, 17], and activation of many apoptosis-associated proteins, such as Raf-1, p21 Wafl/cipl [31]. Most recently, it has been demonstrated that paclitaxel-mediated gene induction is independent of microtubule stabilization [32].

These findings strongly suggest that paclitaxel may cause apoptotic cell death through a gene-directed process. However, current data are not strong enough to draw such a conclusion because paclitaxel-induced apoptosis from other phases of the cell cycle was only occasionally observed, such as under a low concentration or short-time treatment [12, 14]. Baccatin III, due to its lesser effects on microtubule bundling and mitotic arrest, has provided a "window" for us to examine whether this antimitotic agent can cause cell death from other phases of the cell cycle. In both BCap37 and KB cells, 1 μ*M* baccatin III showed significant capacity to induce DNA fragmentation (Figs. 2 and 7) and the typical features of apoptotic cell death (Fig. 5). However, at this concentration (1 μ*M*), little microtubule bundling and few mitotic cells were observed (Fig. 4 and Table 1). As a result, by using time-lapse video microscopy and TUNEL, we were able to observe that a much greater number of apoptotic events occurred in the cells that were not mitotically arrested.

In light of the direct evidence obtained from time-lapse video recordings and the TdT assays, it is quite clear that baccatin III-induced apoptotic cell death, at least in part, occurs via a pathway independent of mitotic arrest. The next question is whether paclitaxel shares the same mechanism with baccatin III. Currently, we do not have solid evidence supporting this issue although the TdT assay has shown that paclitaxel could also cause a low percentage (about 10–15%) of cells to undergo apoptosis from other phases of the cell cycle (see Fig. 7). However, several lines of evidence suggest

that paclitaxel and baccatin III might induce apoptosis by the same mechanism. First, baccatin III and paclitaxel have a similar chemical structure.

From Fig. 1, we can see that paclitaxel is actually composed of baccatin III and an ester side-chain at C-13. This side-chain dramatically enhances paclitaxel's effects on microtubules and is necessary for its full in vitro activity on microtubule stabilization. [18, 33]. Since baccatin III can still cause tumor cell apoptosis, we suspected that the core taxane ring possessed by both paclitaxel and baccatin III may play a critical role in the induction of apoptosis. Second, although baccatin III, at appropriate concentrations, exhibits selective cell-killing activity, higher concentrations of baccatin III (≥ 10 μM in this study) still showed the similar ability to paclitaxel to induce microtubule bundling and mitotic arrest (Table 1 and Fig. 4). This suggests that the core taxane ring is also critical for the promotion of microtubule assembly. Third, both baccatin III and paclitaxel can cause bcl-2 hyperphosphorylation (Fig. 8) which may provide another piece of evidence that baccatin III may induce apoptosis by a similar mechanism to paclitaxel.

Thus, there are structural and functional similarities between baccatin III and paclitaxel that imply that these compounds may be mechanistically similar. Nonetheless, the mechanisms involved in paclitaxel-induced apoptotic cell death appear to be more complex than for baccatin III. The appearance of apoptosis from mitotically arrested cells following paclitaxel treatment may indicate that this is also an important mechanism in the induction of apoptosis. If this is true, then paclitaxel's cytotoxicity may be mediated by coincidental pathways, both G_2/M arrest-dependent and -independent, leading to apoptosis. Further support for these ideas is provided by the results of Lieu et al. indicating that paclitaxel may exert cytotoxicity by mitotic arrest in G_2/M phase or direct induction of apoptosis from S phase [34].

In summary, baccatin III, the synthetic precursor of paclitaxel, was used to evaluate the cytotoxic mechanism of paciltaxel against tumor cells and to determine whether baccatin III and paclitaxel may induce cell death independently of mitotic arrest. The results presented here establish that baccatin III, at suitable concentrations, can induce apoptotic cell death in cells that are not mitotically arrested. Since the structure of baccatin III is a fundamental part of the structure of paclitaxel, the finding of baccatin III-induced apoptosis independent of cell cycle arrest implies that paclitaxel III might induce apoptosis from other phases of the cell cycle by a similar mechanism. In addition, this study suggests that the core taxane ring may be the critical structure for taxoids in inducing apoptotic cell death.

Acknowledgements We would like to thank J. Nicholson for his assistance in image analysis. This work was supported by NIH Grant CA 71851 (to W.F.). M.C.M. is a recipient of a postdoctoral fellowship from the Health Science Foundation of the Medical University of South Carolina.

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