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COMMENTARY

Possible Mechanisms of Paclitaxel-Induced Apoptosis

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ABSTRACT. Paclitaxel, a naturally occurring antimicrotubule agent, has been demonstrated to possess significant cell-killing activity in a variety of tumor cells through induction of apoptosis. It is currently unclear whether this finding suggests a novel mechanism of action for paclitaxel against tumor cells or just represents an end product of the well-known action of paclitaxel on microtubules and cell cycle arrest. Morphologically, a sustained block of mitosis seems to be required for paclitaxel-induced apoptosis because most apoptotic events are observed to occur in cells showing a prior mitotic arrest. However, this morphological correlation alone does not prove that paclitaxel-induced apoptosis is indeed a secondary event resulting from mitotic arrest. Instead, several lines of evidence obtained from recent studies have suggested that apoptotic cell death induced by paclitaxel may occur via a signaling pathway independent of microtubules and G_2/M arrest. BIOCHEM PHARMACOL **57**;11:1215–1221, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. paclitaxel; apoptosis; mitotic arrest; bcl-2; microtubule

Paclitaxel (Taxol®), a plant-derived antineoplastic agent, was originally isolated from the bark of the Pacific yew, *Taxus brevifolia* [1]. This naturally occurring agent has shown great promise in the treatment of a variety of tumors in clinical trials, particularly in drug-refractory ovarian cancer and metastatic breast cancer [2–5]. However, the exact mechanism of the cytotoxicity of paclitaxel against tumor cells is not entirely clear. Previous studies demonstrated that paclitaxel is a unique antimicrotubule agent [6]. Unlike classical antimicrotubule agents such as colchicine and vinblastine that induce microtubule disassembly and/or paracrystal formation [7], paclitaxel acts by inhibiting microtubule depolymerization and promoting the formation of unusually stable microtubules, thereby disrupting the normal dynamic reorganization of the microtubule network required for mitosis and cell proliferation [8, 9]. Therefore, it has been generally believed that the antitumor effects of paclitaxel result from interference with the normal function of microtubules and the blockage of cell cycle progression in late G_2/M phases via prevention of mitotic spindle formation [10].

Since the unique action of paclitaxel against microtubules was discovered in the 1970s [6, 7], much work has been done to characterize the mechanisms by which paclitaxel disrupts the normal function of microtubules and arrests the cell cycle at the G_2/M phase. Little attention has been paid to other possible cellular actions of this antineoplastic agent. In recent years, several laboratories demonstrated that paclitaxel, at clinically relevant concentrations, is able to induce internucleosomal DNA fragmentation and typical morphological features of apoptosis in a variety of tumor cells, including leukemia and human solid tumor cells [11–14]. These results clearly indicate that paclitaxel, in addition to its classical effect on microtubules and cell cycle arrest, may also possess significant cell-killing activity by induction of apoptosis.

Although it has been well recognized that paclitaxel can cause both mitotic arrest and apoptotic cell death, it remains unclear whether paclitaxel-induced cell death is a secondary event resulting from mitotic arrest or represents a novel mechanism of action for paclitaxel against tumor cells. Morphologically, a sustained block of mitosis seems to be required for paclitaxel-induced apoptosis because most apoptotic events have been found to occur in cells showing a prior mitotic arrest [11, 13]. However, several lines of evidence obtained from recent studies have suggested that paclitaxel-induced apoptosis may occur via a signaling pathway independent of microtubule and mitotic arrest. Therefore, the purpose of this commentary is to review these controversial data and to discuss the possible mechanisms by which paclitaxel induces apoptosis.

BIOCHEMICAL AND MORPHOLOGICAL FEATURES OF PACLITAXEL-INDUCED APOPTOSIS

Apoptosis, the terminal event of programmed cell death, is a form of cell death defined by morphological and biochemical characteristics [15, 16]. Apoptotic cell death induced by paclitaxel has been characterized in a number of tumor cells. In general, the biochemical and morphological

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changes occurring in paclitaxel-induced apoptotic cells, such as chromosome condensation, cytoplasmic blebbing, cell shrinkage, and internucleosomal DNA fragmentation, are similar to those apoptotic events induced by other internal or external stimuli [11–14]. However, a unique feature observed in the cells treated with paclitaxel is that apoptotic cell death seems to always occur following a prior mitotic arrest [11, 13]. Typically, when tumor cells are treated with an appropriate concentration of paclitaxel, the first feature noticed is the rapid accumulation of cells arrested at G_2/M phase of the cell cycle. Apoptotic events are usually coupled with mitotic arrest and require 1–2 days to appear. For example, when human breast cancer BCap37 cells were treated with 100 nM paclitaxel, a rapid increase of cells in the late G_2/M phase was observed within a few hours and continued to accumulate until 48 hr. However, the appearance of apoptotic cells was delayed, and most dying cells were observed after 24 hr of drug treatment [13]. Moreover, by using time-lapse video microscopy, nearly all apoptotic events were observed to take place following a prior mitotic arrest [13, 17]. Based on these observations, the cell cycle arrest at G_2/M seems to be required for paclitaxel-induced apoptotis. However, this morphological correlation is not enough to determine if paclitaxel-induced apoptosis is indeed a secondary event resulting from mitotic arrest. Some experiments indicate that paclitaxel-induced apoptosis, under certain circumstances, may occur in a cell without a prior G_2/M arrest. For example, recent studies from several laboratories found that apoptotic cell death induced by low concentrations of paclitaxel may occur without a G_2/M block [18–20], which suggests that paclitaxel-induced apoptosis may take place via a signaling pathway independent of mitotic arrest.

LOW CONCENTRATIONS OF PACLITAXEL MAY CAUSE APOPTOSIS WITHOUT G_2/M ARREST

Early studies indicated that the primary intracellular action of paclitaxel is to specifically bind to cytoplasmic microtubules. This specific binding was demonstrated to be saturable and reversible [8, 21]. Typically, when cells were incubated with [^{3}H]paclitaxel for 1 hr and then washed and cultured in paclitaxel-free medium, the microtubule-bound [^{3}H]paclitaxel was found to be almost completely released after 90 min [8]. Based on this feature, maintaining a certain intracellular concentration of paclitaxel for a definite period of time would be critical to facilitate the cytotoxic action of paclitaxel. However, an experiment conducted by Cheng *et al.* [13] demonstrated that continuous drug exposure is not required for induction of apoptosis. Cell death is triggered as long as the cells are exposed to paclitaxel for only 1 hr. This phenomenon indicates that paclitaxel-induced apoptosis is an irreversible process, which contrasts to the reversible manner of paclitaxel binding to microtubules. Further observations showed that the morphological and biochemical changes occurring in cells exposed to paclitaxel for 1 hr were quite similar to those observed in cells treated with a low concentration of paclitaxel. These changes include "abnormal mitotic exit," micronucleation, and consequently apoptosis without G_2/M arrest, which is consistent with changes previously described by Jordan *et al.* [18]. Therefore, it is possible that the effects of a brief exposure to a high concentration of paclitaxel may resemble those of prolonged incubation with relatively small amounts of the drug.

In recent years, apoptotic cell death induced by low concentrations of paclitaxel has been characterized by several laboratories [18–20]. Jordan *et al.* [18, 22] reported that low concentrations of paclitaxel blocked mitosis of HeLa cells by stabilizing spindle microtubules instead of changing the total mass of microtubules. Cells encountering mitotic arrest in this experimental condition do not resume proliferation or DNA synthesis but enter an interphase-like state, resulting in abnormal mitotic exit and formation of polynucleated cells, which eventually die by apoptosis [18]. Through careful examination of mitotic arrest and apoptosis in HL-60 cells exposed to submicromolar concentrations of paclitaxel, Lieu *et al.* [20] reported that either a low concentration of paclitaxel or a brief exposure to a high concentration of paclitaxel could cause HL-60 cell apoptosis in the absence of mitotic block. Their results indicated that cells treated with 20 nM paclitaxel for 1 hr showed a mitotic block without a subsequent increase in apoptosis, whereas cell treated with 10 nM paclitaxel for 12 hr showed an increase in the apoptotic ratio within several hours without an increase in mitotic arrest. These results indicate that apoptosis does not necessarily result from mitotic block and that these two events can occur independently of each other. In addition, their results also showed that, in addition to the cells in G_2/M phase, the cells in S phase could also initiate apoptosis. Therefore, they concluded that mitotic arrest is not a prerequisite for paclitaxel-induced apoptosis, although they often occur concomitantly. Apoptosis can be triggered in either G_2/M or S phase, which may suggest two different cytotoxic mechanisms of paclitaxel.

SELECTIVE INHIBITION OF PACLITAXEL-INDUCED APOPTOSIS BY GLUCOCORTICOIDS

Another important piece of evidence that supports a separate pathway for paclitaxel-induced apoptosis is the discovery of the selective glucocorticoid-mediated inhibition of paclitaxel-induced apoptosis [12, 17]. The inhibitory action of glucocorticoids on the cell-killing activity of paclitaxel was initially discovered in an androgen/estrogen-induced leiomyosarcoma cell line (DDT1 MF2) in which the cell-killing activity of paclitaxel was repressed dramatically if the cells were pretreated with glucocorticoids [12]. Since growth of this cell line was arrested by glucocorticoids at the G_0/G_1 phase of the cell cycle [23], it had been initially considered that this glucocorticoid-mediated inhi-

bition of paclitaxel-induced apoptosis was due to the prearrest of the cell cycle by glucocorticoids. However, further studies indicated that this glucocorticoid-mediated inhibition of paclitaxel-induced apoptosis was also found to occur in other solid tumor cells, including human breast and ovarian cancer cells [17, 24]. Since growth of these latter tumor cell lines is not affected by glucocorticoids, it suggests that glucocorticoids may directly interfere with the action of paclitaxel. Shortly thereafter, it was found that the inhibitory activity of glucocorticoids on the action of paclitaxel seems to be apoptosis-specific because the effect of paclitaxel on microtubules and cell cycle arrest at G_2/M either was unaffected or was affected only marginally by glucocorticoids. Both flow cytometric and cytospin analyses indicated that the percentage of cells arrested by paclitaxel in the G_2/M phase was essentially the same in the presence or absence of glucocorticoids [17, 24].

This is an interesting phenomenon. Since glucocorticoids do not affect the effects of paclitaxel on microtubules and mitotic arrest, one must wonder about the fate of cells after they are arrested by paclitaxel. Utilizing phase contrast time-lapse video microscopy, the fate of mitotically arrested cells was found to be altered dramatically when tumor cells were pretreated with glucocorticoids. In the group treated with paclitaxel alone, most mitotically arrested cells underwent apoptosis by the end of 72 hr, but in the group with glucocorticoid pretreatment, only a few of the cells underwent apoptosis, while most of the cells passed through mitosis and exhibited micronucleation. This suggests that glucocorticoids produce an effect that is manifested by the inability of cells to enter an apoptotic pathway after mitotic arrest [17]. Mitotic arrest and apoptotic cell death are two major events in cells treated with paclitaxel. The differential response of these two events to the inhibitory action of glucocorticoids has suggested two possibilities: (1) glucocorticoids may specifically disrupt the downstream events of mitotic arrest; or (2) paclitaxel-induced apoptotic cell death may occur via a separate pathway that can be blocked by glucocorticoids.

APOPTOSIS INDUCED BY BACCATIN III IS INDEPENDENT OF CELL CYCLE

The structure of paclitaxel is composed of baccatin III, which contains the core taxane ring, and an ester side-chain at the C-13 position [1, 25]. The side-chain seems to be mandatory for paclitaxel's antitumor activity because baccatin III or its analogs that do not possess the side-chain exhibit less cytotoxic effects on microtubules [26, 27]. In mammalian cells, the bioactivity of baccatin III as an inhibitor of microtubule disassembly was about 50 times less than that of paclitaxel [28]. Recent experiments demonstrated that baccatin III could induce DNA fragmentation and typical morphological features of apoptosis in a number of human solid tumor cells [29] although it requires a higher concentration (about 10-fold higher than paclitaxel). Interestingly, at this concentration, baccatin III has little effect on microtubule bundling and G_2/M arrest. In addition, many apoptotic events were observed to occur in other phases of the cell cycle [29]. When the cell cycle distribution and apoptotic events were analyzed by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and flow cytometric assays, it was demonstrated that the cells undergoing DNA fragmentation appeared to be evenly distributed throughout the cell cycle. These results suggested that baccatin III-induced DNA fragmentation and apoptosis in these cells is independent of the cell cycle (manuscript submitted for publication). It is noteworthy that the morphological features of cells exposed to baccatin III and to low concentrations of paclitaxel are quite similar. However, due to its limited effects on microtubule bundling and mitotic arrest, baccatin III, at an appropriate concentration, provides a "window" so that more apoptotic events are observed to take place at other phases of the cell cycle. Since baccatin III is a fundamental piece of paclitaxel structure, the finding of baccatin III-induced apoptosis independent of cell cycle arrest, on the one hand, implies that paclitaxel and baccatin III may share the same apoptotic mechanism, and, on the other hand, suggests that the core taxane ring may play a critical role in inducing cell death.

THREE POSSIBLE PATHWAYS OF PACLITAXEL-INDUCED APOPTOSIS

Based on the phenomena discussed above and the well-known cellular action of paclitaxel on microtubules and mitotic arrest [8–10, 30], we hypothesize three possible pathways by which paclitaxel could lead to cell death (Fig. 1). The first pathway (indicated by solid lines in the figure) can be considered as the microtubule pathway by which cell death occurs following mitotic arrest. Briefly, paclitaxel, after it crosses the plasma membrane, binds specifically to cytoplasmic microtubules and promotes the formation of unusually stable microtubules. This stabilization effect leads to cell cycle arrest at the G_2/M phase. Eventually, cells arrested at the G_2/M phase may die by apoptosis. In this case, apoptotic cell death is considered as a secondary event resulting from mitotic arrest. The second pathway suggests that cell death induced by paclitaxel is still via the microtubule system, but uncoupled from mitotic arrest. Cells may undergo apoptosis from other phases of the cell cycle. Although microtubules are critical in mitotic function as primary constituents of the mitotic spindle apparatus, they are also important for the performance of many vital interphase functions, such as intracellular vesicular transport, maintenance of cell shape, cellular mobility, and perhaps even transmission of signals from cell-surface receptors to the nucleus [31, 32]. Therefore, when the normal structure and function of the microtubular network are disrupted by paclitaxel, all of the cellular functions associated with the tubulin-microtubule system may be lost or damaged. In cells where this takes place, there may be failure to maintain normal growth and metabolic activities,

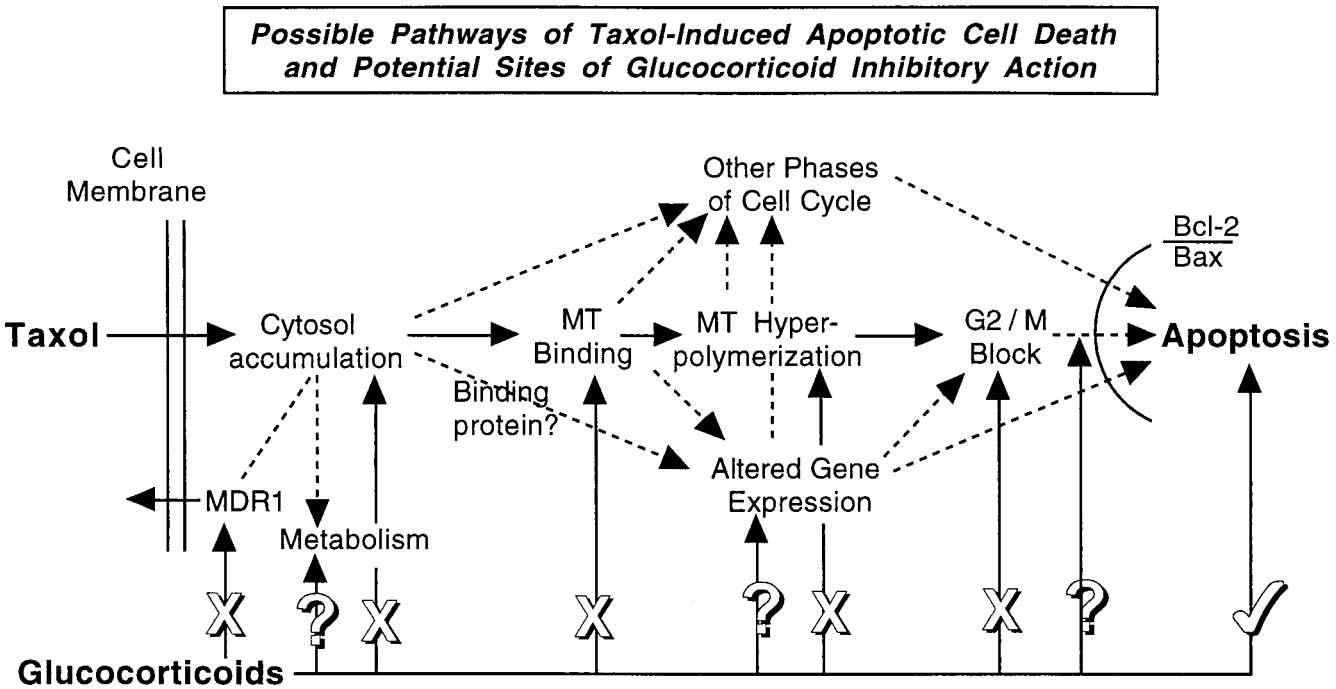

FIG. 1. Hypothesized pathways of paclitaxel-induced apoptosis and potential sites of glucocorticoid inhibitory action. Key: (X) not affected by glucocorticoids; (√) affected by glucocorticoids; (?) remains unclear; and MT microtubules.

and, thereby, cells might also undergo apoptosis even though the cells are not arrested in the G_2/M phase. The third pathway assumes that paclitaxel might exert its cell-killing activity via a pathway completely independent of microtubules. In other words, paclitaxel might cause cell death through a gene-directed process.

Morphologically, most cells exposed to a high effective concentration of paclitaxel seem to undergo apoptosis by the first pathway [11, 13], while apoptotic cell death induced by low concentrations of paclitaxel or baccatin III is more likely to take place via the second or third pathways [18, 20, 29]. Since glucocorticoids only inhibit the cell-killing activity of paclitaxel but do not affect the cellular action of paclitaxel on microtubule bundling and mitotic arrest (see Fig. 1), it provides a unique approach to investigate the molecular basis of paclitaxel-induced apoptosis. Glucocorticoids may specifically disrupt the downstream events if the cell death occurs via the first pathway, or, otherwise, paclitaxel-induced apoptosis is via another hypothesized pathway, either the second or third pathway, and glucocorticoids potentially interfere with one or more steps of these pathways.

DOES MITOTIC ARREST RESULT IN APOPTOTIC CELL DEATH?

If one simply asks whether cells blocked by paclitaxel at the G_2/M phase would undergo apoptosis, the answer would probably be positive. In addition to the morphological correlation of apoptotic cell death with mitotic arrest, many investigators noted that any method that can repress the effects of paclitaxel on mitotic arrest also results in a significant decrease of apoptotic events [33–35]. For instance, Johnson *et al.* [35] found that 5-fluorouracil (5-FU), another antineoplastic agent that can arrest cell growth at the G_1/S phase of the cell cycle [36], could significantly repress the cell-killing activity of paclitaxel in human breast cancer and other solid tumor cells. Further studies indicate that 5-FU actually inhibits the cytotoxic effect of paclitaxel on both mitotic arrest and apoptotic cell death. The mechanism by which 5-FU represses paclitaxel-induced cell death is mainly through preventing tumor cells from entering the G_2/M phase. These results suggest that paclitaxel-induced cell death, at least in part, is cell cycle dependent [35]. Therefore, it seems there is no question that paclitaxel-induced mitotic block can result in apoptotic cell death. The question is whether paclitaxel can also cause cell death through other mechanisms or pathways. For example, if we assume that paclitaxel-induced cell death were a secondary event resulting from mitotic arrest, then the time from mitotic arrest to the onset of apoptosis should be relatively constant, but this period actually varied with each individual cell. Some apoptotic events were observed to occur immediately following mitotic arrest and others occurred much later [13]. Thus, it is possible that apoptotic cell death and mitotic arrest induced by paclitaxel are via two separate pathways, but mitotic arrest requires less time so that apoptosis seems always to occur in mitotic cells.

In fact, some results from early studies have implied that paclitaxel-induced apoptosis may not result from mitotic arrest. Crossin and Carney found that paclitaxel, in addition to its effects on preventing the completion of mitosis,

could also affect the nonmitotic stages of the cell cycle by preventing quiescent cells from re-entering the cell cycle and by promoting tubulin bundling throughout the cell cycle. Interestingly, they found that it was the tubulin bundling rather than mitotic aster formation that correlated with tumor cell toxicity [37]. In addition, through characterization of paclitaxel's cytotoxicity in HeLa 53 cells, Donaldson *et al.* [38] have also suggested that mitotic block might not be a sufficient signal for paclitaxel to induce apoptosis. Instead, their results suggested that paclitaxel-initiated apoptosis might have been via a phosphoregulatory pathway, possibly involving $p34^{cdc2}$ kinase. Based on these results and the fact that paclitaxel-induced mitotic arrest requires less time to occur, it is highly possible that at least some apoptotic events induced by paclitaxel occur via other possible pathways that coincidentally occur in mitotically arrested cells.

PACLITAXEL MAY INDUCE APOPTOSIS THROUGH A GENE-DIRECTED PROCESS

Although there is no solid evidence that paclitaxel-induced apoptosis occurs through a gene-directed process, the possible existence of this pathway has been proposed by many investigators [13, 38–41]. In addition to the features of apoptotic cell death induced by low concentrations of paclitaxel and the selective inhibition by glucocorticoids, a number of apoptosis-associated genes or proteins have been identified to be activated or regulated by paclitaxel. These include genes that act primarily to suppress apoptosis, such as some members of the *bcl-2* gene family [42–44], and genes that may act as effectors of apoptosis, such as the interleukin-1β converting enzyme (ICE) family of proteases [45], and genes that may act as mediators of signal transduction, such as $p21^{waf}$, NF-κB, tumor necrosis factor-α (TNFα), and c-*raf*-1 [39, 46–49]. Bcl-2, the best-known apoptosis inhibitor, has been reported to block apoptotic cell death in a variety of cells [50, 51]. Recent studies have demonstrated that paclitaxel could induce bcl-2 hyperphosphorylation [42]. The phosphorylation of bcl-2 has been postulated to negatively regulate the antiapoptotic effects [43, 52, 53]. The up-regulation of lipopolysaccharide-inducible genes such as interleukin-1β, interleukin-8, and TNFα upon treatment with paclitaxel also has been reported and is probably associated with the ability of paclitaxel to activate NF-κB [46–48], a nuclear transcriptional factor believed to play an important role in coordinately controlling apoptosis [54]. In addition, the tumor suppressor gene p53 was also found to contribute to the biological effects of paclitaxel although the expression of p53 is not directly regulated by paclitaxel. Mutation or inactivation of p53 has been found to affect the sensitivity of some tumor cells to paclitaxel-induced apoptosis [34, 55]. Furthermore, paclitaxel, in the presence of c-*raf*-1 (an upstream regulator of mitogen-activated protein kinase), was reported to induce the expression of p53 and the cyclin-dependent kinase inhibitor $p21^{waf1}$ [39]. Although the discrete roles of these altered genes in paclitaxel-induced apoptosis remain unclear, studies have reported that paclitaxel-altered gene expression is independent of microtubule stabilization [40]. Therefore, it is possible that paclitaxel induces apoptosis via a gene-directed process, i.e. paclitaxel may directly induce or modulate gene expression, which, in turn, triggers the apoptotic process.

CONCLUDING REMARKS

Paciltaxel can cause both mitotic arrest and apoptotic cell death. The mechanism underlying paclitaxel-induced apoptosis and the possible correlation between these two events are not entirely clear. Based on the data discussed above, paclitaxel may induce apoptotic cell death through different mechanisms or apoptotic pathways. In addition to those resulting from the G_2/M block, at least some apoptotic events occur via a signaling pathway independent of mitotic arrest or even the microtubule network. Understanding the mechanism underlying drug-induced apoptosis has been increasingly important in the field of experimental chemotherapeutics. Although the implication of paclitaxel-induced apoptosis in clinical cancer therapy remains to be evaluated, a number of experiments have suggested that the antitumor effect of paclitaxel may be mainly correlated with apoptotic cell death, rather than mitotic arrest [56, 57]. Therefore, elucidation of the molecular basis of paclitaxel-induced apoptosis and its relative contribution to the antitumor effect will facilitate the use of this promising drug, either singly or in combination with other antitumor agents.

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