How Taxol/paclitaxel kills cancer cells

Beth A. Weaver
Department of Cell and Regenerative Biology and Carbone Cancer Center, University of Wisconsin, Madison, WI 53705

ABSTRACT **Taxol (generic name paclitaxel) is a microtubule-stabilizing drug that is approved by the Food and Drug Administration for the treatment of ovarian, breast, and lung cancer, as well as Kaposi's sarcoma. It is used off-label to treat gastroesophageal, endometrial, cervical, prostate, and head and neck cancers, in addition to sarcoma, lymphoma, and leukemia. Paclitaxel has long been recognized to induce mitotic arrest, which leads to cell death in a subset of the arrested population. However, recent evidence demonstrates that intratumoral concentrations of paclitaxel are too low to cause mitotic arrest and result in multipolar divisions instead. It is hoped that this insight can now be used to develop a biomarker to identify the ~50% of patients that will benefit from paclitaxel therapy. Here I discuss the history of paclitaxel and our recently evolved understanding of its mechanism of action.**

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HOW TAXOL WAS DISCOVERED AND RENAMED PACLITAXEL

Between 1960 and 1981, the National Cancer Institute (NCI) and the U.S. Department of Agriculture (USDA) collaborated on a plant screening program that collected and tested 115,000 extracts from 15,000 species of plants to identify naturally occurring compounds with anticancer activity. Samples from a single Pacific yew tree, *Taxus brevifolia*, were obtained by USDA botanist Arthur Barclay on the last day of his expedition in 1962. After his return, crude extracts from bark, twigs, needles, and fruit were tested, and bark extract was found to be cytotoxic. Mansukh Wani and Monroe Wall, working under contract with the NCI at the Research Triangle Institute (Research Triangle Park, NC), received *T. brevifolia* samples in 1964. By 1967, they had isolated and identified the active ingredient from the bark of *T. brevifolia* and named it taxol, based on its species of origin and the presence of hydroxyl groups (Perdue and Hartwell, 1969; Wall and Wani, 1995). In 1971, they published the structure of taxol (Wani *et al.*, 1971), and it entered the NCI drug development program (Table 1).

Taxol showed mixed results in preclinical trials and was not uniformly considered the most promising plant product. The insolubility of taxol in water necessitated its formulation with polyethoxylated castor oil, which can cause severe anaphylactic reactions and further dampened enthusiasm. However, by 1978 taxol had shown efficacy in a subset of mouse tumor models, including P388 leukemia (Fuchs and Johnson, 1978), and it entered clinical trials in 1984 (Walsh and Goodman, 2002a; Tuma, 2003; Table 1).

Several clinical trials were delayed because of a shortage of taxol, the only source of which at the time was the slow-growing *T. brevifolia*. Despite the scarcity, a clinical study on ovarian cancer proceeded and eventually concluded that 30% of patients with advanced ovarian cancer responded to taxol therapy (McGuire *et al.*, 1989). High demand for taxol resulted in severe depletion of *T. brevifolia*, since removing the bark killed the trees. In 1990, the Department of the Interior was petitioned to include *T. brevifolia* on the list of endangered species, and the Pacific Yew Act was passed in 1992 to safeguard the tree (Walsh and Goodman, 1999).

In 1988, it was estimated that the cost of manufacturing taxol from the existing *T. brevifolia* was 10 times the budget available for the project at the NCI, and environmental concerns regarding the long-term prospects for *T. brevifolia* were growing. Owing to the limited accessibility of taxol, as well as its unique structure and cytotoxic potential, at least 30 laboratories worldwide competed to develop a total synthesis. However, because of the complexity of the molecule, these efforts were not successful until 1994 (Holton *et al.*, 1994a,b; Nicolaou *et al.*, 1994). Ultimately, several methods for total synthesis were developed, all of which require roughly 40 steps of reactions, and a more practical, semisynthetic protocol became the

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Address correspondence to: Beth A. Weaver (baweaver@wisc.edu).

Abbreviations used: BMS, Bristol-Myers Squibb; FDA, Food and Drug Administration; NCI, National Cancer Institute.

Year	Event
1964	Sample from Pacific yew tree *T. brevifolia* found to be cytotoxic in joint program between the NCI and the USDA[a,b,c]
1967	Wall lab identifies active ingredient in *T. brevifolia* and names taxol[b]
1971	Taxol structure published[d]
1978	Taxol shows efficacy against mouse tumor models[c]
1979	Horwitz lab shows that taxol stabilizes microtubules[e]
1984	Taxol enters phase I clinical trials[a,c]
1985	Taxol enters phase II clinical trials[a,c]
1991	The NCI selects BMS to commercialize taxol; taxol transitions from public to private property
	First congressional hearing on acquisition of taxol by BMS[a,c]
1992	BMS trademarks the name "Taxol" and assigns new generic name of paclitaxel
	The Food and Drug Administration (FDA) approves Taxol for ovarian cancer
	Pacific Yew Act passed to ensure survival of *T. brevifolia*[a,c,f]
1993	Second congressional hearing on acquisition of Taxol by BMS[a,c]
1994	The FDA approves Taxol for breast cancer
	The FDA approves semisynthetic manufacture of Taxol by BMS[a]
1995	Protection of *T. brevifolia* by Pacific Yew Act ends[a]
1999	The FDA approves Taxol for non–small cell lung cancer (NSCLC)[f]

[a]Walsh and Goodman (2002a).
[b]Wall and Wani (1995).
[c]Walsh and Goodman (2002b).
[d]Wani *et al.* (1971).
[e]Schiff *et al.* (1979).
[f]Tuma (2003).

TABLE 1: How taxol became paclitaxel.

standard approach for production. However, in 1989, no immediate solution for obtaining large quantities of taxol was apparent, and the NCI made the decision to transfer taxol to a pharmaceutical company for commercialization. The request for applications received four responses, and Bristol-Myers Squibb (BMS) was selected in 1991. In 1992, BMS trademarked the name "Taxol" and created the new generic name paclitaxel (Walsh and Goodman, 1999, 2002a,b; Table 1). This occurred despite the fact that the term taxol had been used in >600 manuscripts published over the course of 20 years.

Congressional hearings were held in 1991 and 1993 regarding the transition of taxol to Taxol and paclitaxel (Table 1). The hearings questioned granting BMS a monopoly on a natural resource, as well as the higher price that was charged for drugs, like Taxol, that were identified and developed with federal funding rather than private money. The subcommittee staff concluded that the agreements between NCI and BMS were "not sufficient to fully protect the public interest" (United States Congress, House Committee on Small Business, Subcommittee on Regulation, Business Opportunities, and Energy, 1992). However, the agreements were not substantively altered. Taxol is the most profitable chemotherapy drug in history, and the only drug in clinical use identified by the plant screening program (Walsh and Goodman, 1999, 2002a,b).

BASIC EFFECTS OF PACLITAXEL IN VITRO AND IN VIVO

In 1977, the NCI sent samples of paclitaxel (still referred to as taxol at that point) to Susan Horwitz at Albert Einstein College of Medicine (New York, NY). In 1979, she reported that paclitaxel promotes the assembly of microtubules—polymers composed of repeating subunits of α- and β-tubulin heterodimers. Paclitaxel reduces the critical concentration of purified tubulin subunits necessary for polymerization into microtubules in vitro and increases the percentage of tubulin subunits that assemble. Furthermore, microtubules polymerized in the presence of paclitaxel are protected from the disassembly normally induced by cold or calcium treatment (Schiff *et al.*, 1979). These effects were in stark contrast to previously identified microtubule poisons, including colchicine and vinca alkaloids, which prevent microtubule polymerization (Malawista and Bensch, 1967; Bensch and Malawista, 1968; De Brabander *et al.*, 1981).

Similar to its effects on purified tubulin, paclitaxel promotes microtubule polymerization and stabilization in living cells, where it is capable of antagonizing the effects of colchicine and vinca alkaloids (Schiff and Horwitz, 1980; De Brabander *et al.*, 1981). Phenotypically, paclitaxel treatment arrests a diverse array of cell types in mitosis, in both animal tumor models and cell culture (Fuchs and Johnson, 1978; Schiff and Horwitz, 1980; Milas *et al.*, 1995; Jordan and Wilson, 2004; Orth *et al.*, 2008). A large majority of reports indicate that paclitaxel-arrested cells are in metaphase and contain near-normal, bipolar spindles. Canonical images of paclitaxel treatment show cells with chromosomes aligned at the cell equator, although in some cases one or a few chromosomes have been reported to remain misaligned (Jordan *et al.*, 1993, 1996; Waters *et al.*, 1996). A small number of studies have reported that certain concentrations of paclitaxel induce multipolar spindles (Chen and Horwitz, 2002; Hornick *et al.*, 2008). However, until recently, there was no evidence to suggest that these effects were clinically relevant. The dominant perception, by far, has been that the antitumor effects of paclitaxel occur due to its ability to arrest cells in metaphase on bipolar spindles.

Paclitaxel-induced mitotic arrest occurs due to activation of the mitotic checkpoint (also known as the spindle assembly checkpoint), the major cell cycle control mechanism acting during mitosis to prevent chromosome missegregation. The mitotic checkpoint delays separation of the chromosomes, which enter mitosis as replicated pairs of sister chromatids, until each pair has made stable attachments to both poles of the mitotic spindle. This arrangement ensures that each daughter cell will receive one copy of every chromatid. Chromatids connect to spindle microtubules through their kinetochores, protein complexes that assemble on centromeric regions of DNA. Unattached kinetochores, which have not made stable attachments to microtubules, activate a signal transduction cascade that delays mitotic progression by inhibiting the anaphase-promoting complex/cyclosome (Kops *et al.*, 2005; Lara-Gonzalez *et al.*, 2012; Foley and Kapoor, 2013). Paclitaxel treatment arrests cells in mitosis due to the presence of a small number of unattached kinetochores (Waters *et al.*, 1998).

In addition to its utility in cancer therapy, paclitaxel is also widely used in cell biology. In untreated cells, bipolar attachment of sister chromatids places kinetochores under tension, which helps stabilize the interactions between kinetochores and spindle microtubules. Paclitaxel treatment reduces the tension on kinetochores that maintain bipolar attachment (Waters *et al.*, 1998), and is a useful tool

both for arresting cells in mitosis and for dissecting the contributions of tension versus attachment in satisfying the mitotic checkpoint (Maresca and Salmon, 2010).

CHALLENGES IN STUDYING THE MECHANISM OF PACLITAXEL

A PubMed search for paclitaxel (or Taxol) returns ~25,000 articles. Despite this vast literature, until recently, the clinically relevant concentration for use in cell culture studies has been unclear. There are several reasons for this. First, paclitaxel is dosed at various levels and on different schedules, depending on the disease and the chemotherapy regimen selected. Second, the concentration of paclitaxel in the plasma changes over time as the drug is cleared, primarily by the liver. Third, paclitaxel accumulates intracellularly in cancer cell lines by 50- to >1000-fold, depending on cell type and the concentration added (Jordan *et al.*, 1993, 1996; Yvon *et al.*, 1999). Therefore the concentration of paclitaxel is almost certainly higher in the tumor than in the plasma, where it is typically measured, but there is no linear calculation to predict the fold concentration. Fourth, intratumoral measurements require a biopsy after initiation of therapy, which is not readily accessible outside of a clinical trial.

In the absence of data establishing the intratumoral concentration of paclitaxel, it was reasonable to infer that its antitumor effects were due to mitotic arrest. Unfortunately, determination of the fate of mitotically arrested cells is not straightforward. Mitotic arrest results in either death during mitosis or an abnormal exit from mitosis, without chromosome segregation or cytokinesis, to form a tetraploid G1 cell; this exit is known as mitotic slippage. After slippage, cells can die, arrest, or continue cycling. What determines the fate of cells after mitotic arrest remains unknown.

One factor frequently implicated in the response to mitotic arrest is the mitotic checkpoint. A functional mitotic checkpoint has been reported by numerous groups to be required for efficient cell killing in response to mitotic arrest. In contrast, cells in which the mitotic checkpoint is weakened have also been reported to be sensitive to paclitaxel. Still other studies have found that the state of the mitotic checkpoint does not affect this sensitivity (Rieder and Maiato, 2004; Weaver and Cleveland, 2005; Yamada and Gorbsky, 2006; Ryan *et al.*, 2012). Some have hypothesized that a weakened mitotic checkpoint confers only short-term resistance to mitotic arrest (Janssen *et al.*, 2009), whereas others have proposed that activation of the mitotic checkpoint followed by mitotic slippage results in optimal cell killing (Tao *et al.*, 2005). One popular hypothesis was that the duration of mitotic arrest is predictive of cell death, with cells that arrest longer being more likely to die. However, multiple studies observing individual cells have now shown that the length of time a cell spends in mitosis cannot predict whether it will survive (Gascoigne and Taylor, 2008; Orth *et al.*, 2008; Shi *et al.*, 2008).

The difficulty of predicting sensitivity to mitotic arrest was further demonstrated in a study using nontransformed, chromosomally stable cells. Time-lapse microscopy was used to identify sister cells that resulted from a normal bipolar division without chromosome missegregation. Unexpectedly, the fates of the sister cells in response to mitotic arrest were completely unrelated. If one cell died from mitosis, its sister was no more likely to die from mitosis than it was to slip into interphase (and either die or survive). Thus cell fate in response to mitotic arrest is stochastic and not determined genetically (Gascoigne and Taylor, 2008).

ALTERNATE HYPOTHESIS OF INTERPHASE ACTION

The predominant hypothesis for the past several decades has been that paclitaxel kills tumor cells as a consequence of mitotic arrest. However, despite significant effects on mitosis that are sufficient to cause cell death, it has been suggested that paclitaxel causes death in tumors through effects on interphase cells. This proposal is largely based on the idea that the mitotic index in tumors is not sufficient to explain the efficacy of paclitaxel. Human tumors have a slow doubling time, and calculations that predict mitotic index based on tumor doubling rates, without accounting for cell death, suggest that an insufficient number of cells pass through mitosis in the presence of paclitaxel to account for tumor shrinkage rates (Komlodi-Pasztor *et al.*, 2011, 2012; Mitchison, 2012). However, cell death has been observed in a wide array of untreated patient tumors, and directly measured proliferative rates are much higher than those estimated based on tumor doubling rates (Kerr and Searle, 1972; Kerr *et al.*, 1972; Searle *et al.*, 1973; Lowe and Lin, 2000). In addition, paclitaxel is retained in tumors for >5 d (Mori *et al.*, 2006; Koshiba *et al.*, 2009), permitting an extended window of time for cells to undergo one or more rounds of division in the presence of drug.

Mechanistically, it is unclear how paclitaxel might enact cell death in interphase without having affected a prior mitosis. It has been hypothesized that paclitaxel may interfere with cell signaling, trafficking, and microtubule-mediated transport (Herbst and Khuri, 2003; Komlodi-Pasztor *et al.*, 2011). However, in cell culture, clinically relevant levels of paclitaxel do not cause death in interphase cells that have not previously undergone mitosis in the context of drug (Janssen *et al.*, 2013; Zasadil *et al.*, 2014). Of interest, in tumor models observed using intravital microscopy, the mitotic index after treatment with doses of paclitaxel expected to cause mitotic arrest was quite low (Orth *et al.*, 2011; Janssen *et al.*, 2013), leading to the suggestion that the microenvironment allows paclitaxel to exhibit interphase effects not observed in culture. However, no clear cytotoxic mechanism has yet emerged.

CLINICALLY RELEVANT CONCENTRATIONS OF PACLITAXEL CAUSE MULTIPOLAR DIVISIONS

To better mimic the antineoplastic effects of paclitaxel in cell culture, we first collaborated with our physician colleagues to design a clinical trial to measure the intratumoral concentration of paclitaxel in primary breast tumors (Zasadil *et al.*, 2014). To remove as many confounding variables as possible, patients who had not received prior therapy and did not require concurrent therapy were enrolled. At 20 h after the initiation of the first dose of 175 mg/m^2 paclitaxel, samples were obtained to measure paclitaxel concentration in both plasma and tumor. The 20-h time point was selected because the mitotic index of breast cancer cells in culture is increased ≥15-fold between 16 and 32 h after paclitaxel administration, and we therefore predicted that mitotic arrest would be evident at this time point. To assess whether tumors responded, measurements were obtained by ultrasound and/or mammogram before treatment and after four standard cycles of paclitaxel.

As predicted by prior cell culture experiments, the intratumoral concentration of paclitaxel (1–9 µM) was higher than the plasma concentration (80–280 nM) in all patients. However, contrary to expectations based on cell culture data, mitotic arrest was neither necessary nor sufficient for tumor shrinkage in response to paclitaxel (Zasadil *et al.*, 2014).

As a second step in determining the appropriate dose of paclitaxel with which to treat our cultured cells, we determined the extent to which the drug was concentrated in breast cancer cell lines. Consistent with previous results (Jordan *et al.*, 1993, 1996; Yvon *et al.*, 1999), we found that paclitaxel accumulated to a differing extent in distinct cell lines. High-performance liquid chromatography analysis determined that treatment with low nanomolar concentrations of

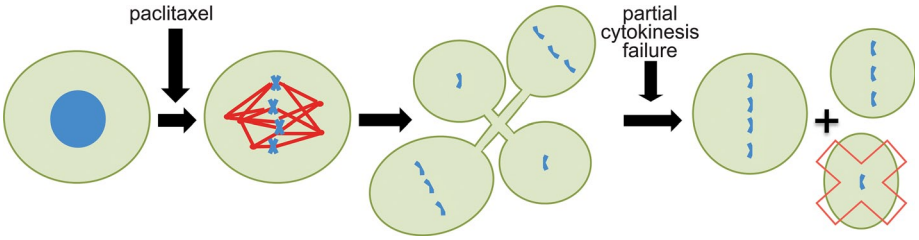

FIGURE 1: Clinically relevant concentrations of paclitaxel kill tumor cells by inducing multipolar divisions. Cells entering mitosis in the presence of concentrations of paclitaxel equivalent to those in human breast tumors form abnormal spindles that contain additional spindle poles. Rather than mounting a long-term mitotic arrest, these cells enter anaphase and divide their chromosomes in multiple directions. However, a portion of the cytokinetic furrows often fail, and two or three daughter cells are usually produced. Chromosome segregation is randomized due to multipolar division followed by partial cytokinesis failure. The resultant daughter cells are aneuploid, and a portion of these die (red X), presumably due to loss of one or more essential chromosomes.

paclitaxel (5–10 nM for MDA-MB-231 and 10–50 nM for Cal51) resulted in clinically relevant intracellular concentrations of 1–9 μM.

Of interest, whereas higher concentrations of drug cause a robust mitotic arrest in these breast cancer cell lines, clinically relevant concentrations do not. They do, however, induce multipolar spindle formation. Importantly, a majority of mitotic cells in patient tumors treated with paclitaxel also exhibit multipolar spindles. After a brief delay, cultured cells in clinically relevant concentrations of paclitaxel proceed through mitosis on multipolar spindles and often segregate their chromosomes in three, four, or five different directions. However, a portion of the cytokinetic furrows usually fail, and most divisions in paclitaxel produce two or three daughter cells (Figure 1; Zasadil *et al.*, 2014).

IMPLICATIONS

Because paclitaxel causes mitotic arrest at concentrations typically used in culture, and was believed to do so in human tumors, numerous other drugs that induce mitotic arrest without affecting microtubule dynamics have entered clinical trials. These include inhibitors of Aurora A, CENP-E, Eg5/KSP, and Plk1. The expectation for these drugs was that they would have the efficacy of paclitaxel without one of its major dose-limiting toxicities—peripheral neuropathy. Peripheral neuropathy after paclitaxel therapy is presumed to result from impaired transport along the longest axons in the body, although data for this are scarce, and some chemotherapy drugs that do not affect microtubules, such as cisplatin, cause the same symptoms. Disappointingly, despite causing an accumulation of mitotic figures, the new classes of antimitotic drugs have yet to match the efficacy of paclitaxel (Chakravarty *et al.*, 2011; Komlodi-Pasztor *et al.*, 2011; Mitchison, 2012). This may be because the cytotoxic effect of paclitaxel in patient tumors is induction of multipolar divisions rather than mitotic arrest.

Numerous screens to identify markers of resistance or sensitivity to paclitaxel have been performed. These have identified a diverse array of candidates, including proteasome subunits, cyclin G1, and solute carrier genes (Rouzier *et al.*, 2005; Swanton *et al.*, 2007; Whitehurst *et al.*, 2007; Pusztai *et al.*, 2009; Juul *et al.*, 2010; Wertz *et al.*, 2011; Njiaju *et al.*, 2012; Russell *et al.*, 2012). However, these discoveries have not yet led to a validated biomarker that predicts which patients will benefit from paclitaxel therapy. This may be due, at least in part, to an emphasis on higher concentrations of drug that cause mitotic arrest and rapid cell death. It is hoped that recognition of the clinically relevant mechanism of paclitaxel will facilitate identification of a biomarker capable of predicting which patients will benefit from its use.

CONCLUSION

Like all drugs, paclitaxel exhibits concentration-dependent effects. It is not surprising that the rapid, dramatic effects of higher paclitaxel concentrations on mitosis and cell death were originally believed to be responsible for its efficacy in cancer therapy. Unfortunately, the barriers to acquisition of patient samples by basic scientists substantially delayed the finding that lower concentrations, which are slower to evoke cell death, are clinically relevant. However, newly available data demonstrate that, rather than causing mitotic arrest, intratumoral concentrations of paclitaxel cause cell death due to chromosome missegregation on multipolar spindles. It is hoped that, in addition to expediting identification of a predictive biomarker for paclitaxel treatment, this insight will also encourage collaboration between basic scientists and clinicians.

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