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REVIEW

Paclitaxel (Taxol): From Nutt to Drug

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Paclitaxel (Taxol), a mitotic inhibitor, is a natural diterpene ester isolated from the stem bark of the pacific yew tree (Taxol is the registered trade name for a formulated drug based on the chemical paclitaxel and marketed by Bristol-Myers Squibb Co., U.S.A.). It has been shown that the anticancer [61], antineoplastic [21], and antifungal activities [87, 88] are due to microtubule stabilizing activity and potent cytotoxicity. To date, taxol has been isolated from quite a few *Taxus* sp. such as *T. brevifolia*, *T. baccata*, *T. cuspidata*, *T. canadensis*, *T. chinensis*, *T. x media*, *T. floridana*, *T. yunannensis*, *T. mairei*, *T. sumatrana* and *T. wallichina* [7, 8, 16, 21, 26, 29, 36, 47, 48, 89, 100]. Taxol was also discovered from the needle and bark of *Austrotaxus spicata* and *Torreya grandis* [52], and from the endophytic fungi *Taxomyces andreanae* [81], *Pestalotiopsis microspora* [84], and unclassified fungal species from the *Taxus yunnanensis* [69]. Recently, a taxol-producing bacterium *Erwinia* sp., which was isolated from *T. canadensis*, was reported by Page *et al.* (1996. US patent 5,561,055).

Although, taxol has been called "the best anticancer agent" by NCI (National Cancer Institute) among the 13 marketing approved natural anticancer drugs during the last fifteen years, taxol also has some pharmacological drawbacks in that it is difficult to inject into the body, has low solubility, high cost, adverse side effects, and is needed in high dosage. Efforts of various research fields in overcoming these and other problems are summarized in Table 1.

Actually, taxol is not a cure for cancer but can extend the patient's life by up to 6 to 14 months [15, 61, 86]. In cost effective analysis with cisplatin/cyclophosphamide (C/C), a standard therapy, and cisplatin/taxol (C/T), the mean survival duration is increased from 2.06 years with the standard therapy to 2.44 years with taxol combination. The taxol therapy is more expensive, with a mean cost of $17,469 (Canadian) per patient treated with C/T compared with $5,228 per patient with C/C. The incremental cost effectiveness ration is $32,213 per year gained [15]. To reduce the clinical cost and implement wide usage, an economical mass production of taxol is necessary. In addition, obtaining a large-scale and stable supply of taxol is another old dilemma. Since December 1992, when taxol was appoved by the FDA (Food and Drug Administration) for use in the USA for the treatment of refractory ovarian cancers, obtaining and generating a reliable supply of taxol have been at the forefront of research fields such as plant biotechnology, chemistry, microbiology, cell biology, genetic engineering, and pharmacology. This is due to the fact that the only commercial avaiable source of taxol was the bark of *Taxus brevifolia*, which is an endangered species. Unfortunately, this tree is relatively rare and exhibits very slow growth and extremely small yields of taxol (<0.02% dry weight). It takes about 10,000 kg of bark to make only 1 kg of taxol and co-produced or co-extracted similar compounds such as BIII (baccatin III), 10-DAB (10-deacetylbaccatin III), and CM (cephalomannin), do not even show antitumor activity. However, clinical treatment with taxol is composed of 10 or more courses; one course uses 125~300 mg of taxol. In the USA, 12,000 women die by ovarian cancer per year. Therefore, the estimated taxol required for ovarian cancer therapy alone is about 36 kg. In considering the approval for treatment of breast cancer, and other refractory cancers including head, neck, and non-small cell lung cancers in clinical

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Table 1. Problems and solutions relating to taxol production in various research fields.

Problems	Research Fields	Solutions	Reference
Supply shortage High cost Environmental destruction	Organic chemistry	Total synthesis of taxol and taxol skeleton	13, 37, 62
		Semi-synthesis of taxol	42, 61
		Development of beneficial analogue	61
	Plant biotechnology	Development of stable plant cell line	
		Development of rapid growth and high yielding cell line	77
		Use of alternative and renewable plant source	52, 77
		Development of production method and process	19, 67
		Development of recovery process	54, 70, 71
		Tissue and suspension culture	46, 93
		Hydroponical culture, Nodule culture	16, 17, 94
	Pharmacology	Combined use of other drug and radiation	6
		Sequential administration	15
		Use of beneficial analogue	41
	Microbiology	Screening of taxol producing strain	69, 81, 84
		Optimization of fermentation conditions	81
		Large-scale fermentation	*CPI
	Cell biology Biochemistry Genetic engineering	Elucidation of taxol biosynthesis mechanism	12, 49
		Elucidation of tumoricidal mechanism	2, 6, 51, 65
		Signal transduction in plant cell	
		Transformation of *Taxus* sp.	32
		Cloning of taxol synthesis gene family	95
Side-effects	Organic chemistry	Drug design: more potent and soluble potent modifier	63
Low solubility High dosage	Plant biotechnology	Production of new taxane	8, 100,102
	Pharmacology	Use of protection factor	44, 72
		Combined use of other drugs	
		Advanced drug-delivery system (DDS)	97, 101
Stability Binding affinity		Development of stabilization method in culture medium and aqueous state	91, 103
		Nonspecific and specific adsorption	78
		Molecular interaction	1

*CPI (Cytoclonal Pharmaceutics Inc., U.S.A.).

trials [27, 40], the demand of taxol may exceed 300 kg or 750,000 trees per year.

As expected in its structure, the low solubility of taxol (4 mg/l in water at room temperature) is another problem. Taxol could self-associate by intermolecular interaction in organic solvents or in high concentrations [1]. For solubilization, the current drug formulation contains polyoxyethylated castor oil and dehydrated ethanol which brings about hypersensitivity and leaching from the infusion bags [91]. Also, it requires in-line filters with hydrophobic, microporous membranes and 24 h infusion for safe intravenous administration. Recently, an advanced drug-delivery system (DDS) was developed by several researchers. Winternitz *et al.* [97] developed a polymeric surgical paste formulation for taxol delivery, and Zhang *et al.* [101] made another polymeric micellar taxol, which showed promise for the useful chemotherapeutic formulation. Prodrugs for oral administration and stable oil-in-water emulsion using safflower and lecithin were granted U.S. patents (Hostetler *et al.*, 1996, US patent 5,484,809, and Kaufman *et al*, 1997, US patent 5,616,330). In spite of the above-mentioned problems, however, taxol is one of the most significant cancer therapies available today, now available in more than 50 countries. In this review, we share the literature survey information of taxol production, biosynthesis, and commercial application.

Taxol and the Yew Tree

Taxol is a diterpenoid alkaloid characterized by a tetracyclic taxane molecular core with an *N*-benzoyl-β-phenylisoserine side chain at the C-13 position [90]. The structure and physicochemical properties of taxol are summarized in Table 2. Although taxol research was started in 1962 by Arthur Barclay of the US Department of Agriculture with the natural resources screening program in NCI, Monroe Wall and M. C. Wani, the chemists at the Research Triangle Institute (RTI) in North Carolina found antitumor activity against a broad range of rodent tumors from *Taxus brevifolia* Nutt in

Table 2. Physicochemical properties of taxol.

IUPAC systematic name	[2aR-(2a,4,4a,6,9,11,12,12a,12b)]-Benzoylamino-hydroxy benzenepropanoic acid 6,12b-bis(acetyloxy)-12-(benzoyloxy)-2a,3,4,4a,5,6,9,10,11,12,12a,12b-dodecahydro-4,11-dihydroxy-4a,8,13,13-tetramethyl-5-oxo-7,11-methano-1H-cyclodeca[3,4]benz[1] ester
Molecular formula	$C_{47}H_{51}NO_{14}$
Molecular weight	853.92 g/mol
% composition	C 66.11%, H 6.02%, N 1.64%, O 26.23%
Solubility in water	4 mg/l at room temperature
Melting point	213~216°C
Specific rotation	-49° in methanol
TLC	R_f=0.125 (silica 50% ethyl acetate in hexanes)
UV max	227, 273 nm in methanol
IR (thin film)	V_{max}: 3472, 2937, 1720, 1652, 1520, 1241/cm
Mass spec	*m/e*=854.3360 M+H calculated for $C_{47}H_{51}NO_{14}$=854.3388

1966. Through the efforts of Monroe Wall, taxol was obtained in pure form in 1969 and its structure was elucidated in 1971. However, NCI was not interested in his result and Wall gave up without patenting his work. In 1977, the first clinical trials against the human xenography system and murine B16 melanoma cells were achieved. After discovery of the unique mode of action of taxol by Schiff *et al.* in 1979, taxol was highlighted by many researchers [3]. In 1983, the results of the first human trials were obtained, which showed great promise for incurable ovarian cancer, as reported by Johns Hopkins University in Baltimore in 1989. Due to these results, the cooperative research and development agreement between the NCI and BMS became possible. Finally, taxol was appoved for the treatment of refractory ovarian cancer by the FDA for use in the USA in December 1992, and also by Sweden in July 1993. Due to the successful result of anticancer activity over breast cancer, FDA approved the use of taxol for recurrent breast cancer in April 1994. In June 1994, the 3 h infusion method was also approved by the FDA to reduce the incidence of neutropenia, a disease in white blood cells related to susceptibility to infections. Currently, clinical trials are continuing on other forms of cancers including head and neck cancers. Despite the rapid advance in the clinical field, the supply of taxol still remains an unsolvable problem. Although the production of taxol through the semisynthetic method from 10-DAB or BIII for clinical use was approved by the FDA, and total chemical synthesis was successfully achieved [13, 37, 63], the commercial source of taxol is still from the inner bark of the yew tree.

Research on the yew tree still continues for taxol production as well as preservation of endangered species. The pacific yew tree, *Taxus brevifolia*, is a conifer from the family Taxacerae. The US Forest Service estimates that 130 million yew tree grow on 1,778,000 acres of National forests in the Washington and Oregon cascade and Oregon coast range [11]. The Pacific yew tree grows to approximately 50 feet in height and reaches a diameter of around 2 feet. It has an irregular and twisted trunk that bears purplish-brown bark. Its needles, which are flat and flexible, are dark green on top and light green underneath. The yew tree is unisexual and shade tolerant; it cannot survive and grow without an abundance of shade. It does not reproduce using cones unlike other conifers, but bears seed, singly. Its growth is very slow, about 8 inches per year, almost one-tenth as fast as other conifers. Thus, the growth of yew tree is not easily controlled. However, as recently reported by Mitchell *et al.* (the Pacific Forestry Centre of the Canadian Forest Service), the pacific yew can be successfully propagated and grown with minimal cost by seedling transplants and rooted cuttings. Although subsequent growth is slow in the first year, survival rates were around 70% when the plants were kept at high humidity.

Another interesting tree is the European yew, *Taxus baccata*, which is more abundant than Pacific yew. It contains taxotere, which is more soluble and efficient than taxol in killing lab-grown cancer cells. The taxotere in European yew is ten times more concentrated than the taxol found in the Pacific yew. Last year, taxotere, which is synthesized from the precursor 10-DAB in the needles of the European yew tree *Taxus baccata*, was approved for the treatment of metastatic breast cancer by the FDA. Singh's group also reported an efficient regeneration method for *T. baccata* [77].

A garden variety of the yew tree, *Taxus x media* Hicksii, a hybid of *T. baccata* and *T. cuspidata*, which has needles with high taxol yield and rapid growth, is being raised commercially in Mississippi. Other *Taxus* sp. such as Eastern yew, *Taxus canadensis* and *Taxus*

chinensis have been also intensively cultivated and investigated as taxol sources.

Biosynthesis and Secretion of Taxol

Taxol is a secondary metabolite, which is produced by specialized or differentiated tissue; most notably by bark in the case of taxol, or by leaves in the case of baccatin. Production of secondary metabolites such as taxol is influenced by a wide array of external stimuli. Until recently, little was known about taxol metabolism, biosynthesis, storage, transport, release, and degradation. Although some mechanisms of taxol production and/or secretion in cell cultures may differ from those of whole plant cells, we have obtained our knowledge from cell culture systems. Biosynthesis of the taxane skeleton does not involve the mevalonate pathway, while acetate added contributes to the acetyl side chain of taxuyunnanine C [2,5,10,14-tetra-acetoxy-4(20),11-taxadiene] but not to the taxane ring system [14]. The taxane skeleton is synthesized from GGDP (geranylgeranyl diphosphate) cyclization by taxa-4(5),11(12)-diene synthetase, including extensive oxygenation of a diterene olefin intermediate with putative NADPH and O_2-dependent microsomal cytochrome P450 oxygenase [12, 49]. GGDP is synthesized from IPP (isopentenyl pyrophosphate) in the cytoplasm and plastids of the plant tissues [79]. The distribution of taxadiene synthetase in *T. brevifolia* is mostly in the peeled bark and adhering cambium cells, which coincides with the distribution of taxol in Pacific yew tissue [20, 83]. Taxadiene synthetase is very similar to diterpene cyclases such as abietadiene synthase in common gymnosperms, in sequence homology (46% identity, 67% similarity with a diterpene cyclase from grand fir), molecular weight (79,000), and divalent metal ion requirement (especially Mg^{++}). However, the activity of taxadiene synthetases of the yew tree is much lower than the activity of other terpenoid cyclases, and reaction intermediates are found only at trace levels (5~10 μg/kg-bark). To date, the taxane skeleton synthesizing step is considered to be the rate-limiting step. Interestingly, taxadiene synthetase in the Pacific yew, which has an unusual optimum pH of 8.5, is not inducible by stem wounding or elicitor treatment unlike the inducible abietadiene synthase [35]. Thus, increased taxol production by elicitation is not directly related to the activity of taxadiene synthase. This enzyme was recently identified and cloned to produce hybrids that showed higher taxol yields [95, and Suffness, 1995, "Taxane anticancer agents", ACS symposium. 1-17]. After the oxygenation step of the taxane skeleton [34], 10-DAB may be subject to an acetylation step. Zocher *et al.* [103] produced BIII from 10-DAB with acetyl coenzyme A from a crude extract of *Taxus baccata*. Purification and characterization of the acetylation enzyme from sapling extract has been conducted by this group. After attaching the side chain as phenylisoserine into BIII, benzoylation of phenylisoserine, which is synthesized from phenylalanine via β-phenylalanine, occurs. According to Fleming *et al.* [25], this is due to direct incorporation without degradation and reorganization of phenylisoserine. However, Srinivasan *et al.* [79] reported that taxol is synthesized only in plastids from plastidic IPP in *Taxus chinensis* cell culture, while BIII is synthesized in the cytoplasmic and plastidic compartments, and therefore BIII may not be a direct precursor of taxol. It suggests that the taxol synthesis system is different from that of BIII in *Taxus* sp. cell cultures although some pathway are held in common. After taxol is made, it is secreted by a specific excretion system localized in specific sites in the cell. Until now, the secretion system has not been identified. Wickremesinhe *et al.* [94] and Fett-Neto *et al.* [21] reported that the levels of taxoids found in culture media were usually less than 10% of the total amount found in the *Taxus x media* cells. But Srinivasan *et al.* [80] and Hirasuna *et al.* [36] reported that more than 90% of the taxol was secreted to the culture media in cultures of *T. baccata*. This difference may be due to cell culture conditions, cell lines used, media composition, and etc. Plant tissue research has shown taxol to be localized almost exclusively in the cell wall of phloem, vascular cambium, and xylum [16, 73], not sequestered in the vacuoles plastids and associated tannin [18]. This result was confirmed by xylanase treatment of protoplasts from *Taxus* sp, where taxoids recovery from the culture medium was 300% higher than from non-enzyme-treated cells. However, it is unclear whether taxol itself is excreted across the cell wall or whether its precursors are excreted and then modified. An efficient stabilization and secretion method, as well as in-situ recovery for intracelluar taxol, could make use of the fact that taxol is degraded to first order in cell cultures (Roberts and Shuler, 1996, American Institute of Chem. Eng. Chicago. 158), taxol and BIII are easily deacetylated in mild alkaline state [103], and Ca^{++} is strictly relative to taxol partitioning.

Unique Mechanism of Taxol

Unlike other antimicrotubule agents, such as cholicin, podophyllotoxin, combretastatin, and vinca alkaloids, taxol has microtubule stabilization activity and potent cell cytotoxicity. This unusual stability inhibits the normal dynamic reorganization of the microtubule network, that is, it blocks the cell's ability to disassemble the mitotic spindle during cell division. As a specific receptor in the cell membrane (CM) for taxol transport has not been reported, taxols ease in penetrating the CM could be due to its low molecular weight and hydrophobicity. In fact, adriamycin and taxol-resistant

cell lines such as K563 cells show different cell membrane composition to sensitive counterpart cells, and in cultures without taxol, the fatty acid signals were partially recovered [50]. Taxol crossing the CM would bind β-tubulin directly, and preferentially form microtubules rather than tubulin dimers or β-tubulin only [38, 65]. Also, by examination of the fluorescent derivative of taxol, the aromatic ring at C-2 on taxol appear to contact the protein in its complex with microtubules, and at least one of the four tryptophane residues of β-tubulin is in close proximity to the taxol binding site. Normal microtubules consist of 13 protofilaments which are in linear strand form made up of α and β tubulin dimers. Assembling the tubulin into microtubules requires GTP and MAP (microtubule associated proteins), which may improve microtubule rigidity by bridging protofilaments. However, taxol changes this reaction in favor of microtubule formation. Taxol decreases the critical concentration of tubulin required for microtubule assembly in the presence or absence of factors that are usually essential for assembly, such as exogenous GTP and MAP [31]. Formation of microtubules with taxol is maximum at a taxol: heterodimer of tubulin ratio of 1:1 and which has less rigidity due to lack of cross-linking in protofilaments by MAP. As a result, taxol induces G_2/M arrest in the cell division cycle [38] and finally brings about cell death. Since tumor cells divide more rapidly than normal cells, the target for taxol will mainly be tumor cells. But other rapid-dividing normal cells are also affected by taxol and this can bring about severe side-effects; normal cells treated with taxol *in vitro* lose some of the functions of the microtubule networks such as chemotaxis, migration, and cell spreading.

However, the potent cytotoxicity of taxol is not only due to microtubule stabilizing activity. On removal of taxol, treated cells could exit the abnormal cell cycle. But, cells treated with taxol did not grow and finally lost viability, including severe DNA fragmentation of approximately 200 bp by an unidentified cytotoxic mechanism [9, 27, 68]. Until now, it has been estimated that INF, TNF, IL-8 and other cytokines induced by taxol via specific intracellular secondary signals, were related to this cytotoxicity, and that increased bcl-2 phosphorylation [2], tyrosine phosphorylation, and macrophage tumoricidal activity due to taxol bring about tumor cell death [6, 51]. Also, at least 12 taxol-induced gene families were reported by Cheng *et al.* [9] and transformation by T-DNA of *Agrobacterium tumefaciens* was succesfully conducted in *T. baccata* and *T. brevifolia* [32].

For the elucidation of the mechanism and synthesis of a more potent taxol analogue, research on the structural relativity and biological activity has been conducted by many researchers. According to their reports, the side chain of taxol is critical for maintaining activity. In particular, protection of the C-2-OH group as an ester results in a loss of activity in terms of microtubulin stabilization but not in cytotoxicity [96]. The C-3-amide-acyl group is critical but may be aromatic or alkyl in nature. C-3 bound nitrogen can be replaced by an oxygen atom without loss of activity. The C-3 aryl group is needed, and replacement by a methyl group reduces the activity 19-fold. In the taxol skeleton, the oxetane ring is crucial for maintaining the activity, and ring opening leads to a dramatic decrease in bioactivity. Removal of the C-10 acetyl group does not affect the activity while removal of the C-2-O-benzoyl group causes a drastic reduction in activity. With these results, it is possible to design a super-taxol and its derivatives.

For a while, a taxol-mimicking agent was reported by several researchers. TALP (taxol-like protein) from human term placenta (35 kD) is very similar to taxol, having potent microtubule stabilization activity via direct binding of microtubules, in spite of the absence of GTP. When TALP (0.5 μM) was treated with taxol (10 μM), the lag-time for microtubule polymerization was shortened and showed hyperbolic kinetics [41]. Another amazing thermostable protein, called Ensconsin, showned unique microtubule stabilization activity. Unlike taxol, it binds tubulin with 1:6 ratio [5]. Also, like Taxol, Epothilons A and B from the culture broth of *Sorangium cellulosum* [28], and Lapachol from a dichromethane extract in *Kigelia pinnata* [39] show antifungal activity and cytotoxicity for animal cells. Development of these kinds of agents would contribute to solving problems of short supply and pharmacological drawbacks.

Pharmacokinetics and Side-Effects of Taxol

Significant hepatic metabolism of taxol was found in rats and humans. 6-alpha-hydroxytaxol was found to be the principle human metabolite of taxol [33]. Urinary excretion is insignificant (5~10%), but larger amounts of taxol are found in bile (about 20% in humans). The metabolites were as active as taxol in preventing cold microtubule disassembly, but were 9 to 39 times less cytotoxic when tested *in vitro* on L1210 leukemia cells [59]. Taxol elimination appears to be bi-exponential with a terminal half-life of 1.5~8.4 h. On the other hand, adverse side effects from taxol have been reported similar to other anticancer agents. In 1993, 43 cattle which had ingested 0.36~0.7 g of fresh *T. baccata* plant/kg of body weight died within four hours [64]. In clinical use, taxol shows some side effects such as hair loss, mouth sores, and reduction in the infection-fighting white blood cells, because taxol has an effect on bone marrow. Anemia, and bone and muscle pain were also common side effects. If growth factors such as a granulocyte colony stimulating factor or NGF are used,

the bone marrow is protected [44, 72]. Also, taxol is not irreversibly toxic to hematopoietic stem cells. The most common cardiac toxicity is a transient asymptomatic bradycardia [55]. A more unusual problem is that of hypersensitivity which occurs in about 1/4 of unprepared patients. This includes difficulty in breathing, itchiness of the skin, and low blood pressure. It was later determined that the polyoxyethylated castor oil (cremophor EL), in which taxol is prepared, was responsible for these effects [91]. Recently, Perry *et al.* [66] reported that taxol may be related to CNS toxicity with symptoms of recurrent headache and ataxia.

Production of Taxol: Old Problems and New Approaches

Extraction of taxol from natural sources. *Destructive harvest* and *bark extraction*: Taxol is contained in all parts of *Taxus* sp. plant tissue including bark, shoots, foliage, needles, and roots. Among them, the inner bark is used for taxol extraction due to higher accumulation although there is a difference according to species, location, and age [60, 85]. The current isolation methods starting from the milled bark of *T. brevifolia* yield 0.01~0.014% taxol of the extracted dry weight. The dried inner bark is extracted with organic solvents in three steps followed by a four-step chromatographic process (Castor, 1995, US patent 5,440,055). A new method, with a two-step organic solvent extraction and C18-silica reverse phase chromatography and decolorization/filtration process for pilot-plant scale extraction, provided a taxol yield of 0.04% and 75~80% recovery [71]. For removal of cephalomannine, ozonolysis was used instead of the chemical method using osmium tetroxide, although this step had lower yields. However, current extraction methods encounter several problems such as low yields, environmental concerns, high costs and long times - 18 to 24 months are required for harvesting, drying and processing. Therefore, the development of plant strains with rapid growth and higher content of taxol is the main concern in plant technology. Intensive cultivation of *Taxus* sp. has been conducted by Weyerhauser Co. with support from NCI, BMS, and several pharmaceutical companies in the USA since 1987. For a stable supply and preservation of yew trees, partial bark removal from one side of the tree was reported to not seriously affect the growth of the tree [56]. Also, nodule culture was conducted by Ellis *et al.* [16], and hydroponic growth of *Taxus* plants using Johnson's nutrient solution was successfully achieved for 22 weeks [94].

In a storage effect, there were controversial results. After 9 days of storage at 22°C, taxol concentrations were 40% and 26% at 4°C, higher than that of fresh harvest materials. About 9 days of storage is necessary for taxol extraction [74], but ElSohly *et al.* [19] reported no apparent change of taxol concentration stored at 4°C/20°C and stored intact/powdered for 10 weeks. Since it usually takes from 18 to 24 months from forest to drug, we need information on long-term storage effects.

Leaf extraction: Growing foliage, a renewable source, in nurseries is a viable option as taxol is contained in all species of yews and in all parts of the plants. It would provide security and quality control, but taxol from foliage is not yet commercially licensed and has the problem of labor-intensive collection. Also, the needles have a higher wax component and 7-epi-10-deacetyltaxol than stems [54]. Recently, however, taxol was successfully obtained from needles of *T. x media* and *T. floridana* in a large-scale process by Rao *et al.* [70] with taxol yields between 0.012~0.015% from *T. x media* and 0.01% taxol and 0.06% 10-DAB from *T. floridana*. Before long, new taxanes were continuously reported from the various *Taxus* sp. [43, 102]. Unfortunately, the antitumor activity of the new taxanes has not measured in most cases.

Chemical synthesis (semi, or total synthesis) and design of super-drug. Although taxol has an extremely complex structure and 11 stereocenters, the total chemical synthesis of taxol has been conducted by the Holton group from (-)-borneol, a 45-step linear process which yielded 4~5% taxol [4, 37]. Another total synthesis was achieved by the Nicolaou group, a 32-step process which yielded only 0.054% [4, 63]. Recently, Danishefsky *et al.* also reported their route to taxol which started with the Wieland-Miescher Ketone in 1996 [13]. Until now, total synthesis has not been considered for the mass production method because of complicated multi-reactions and low economical feasibility. However, many organic chemists have tried to not only develop newer and shorter routes to taxol, but also with a view to creating a modified structure that is more active. Taxol beneficial analogs, combinations of natural precursors and chemical synthesis, have been produced as a more potent anticancer drug with fewer adverse side effects, but the process is costly [61]. Synthesis of the taxane skeleton alone, involving 13 steps, was reported, making it the shortest route from readily available inexpensive starting materials (Magnus, 1996, US patent 5,508,447). Another acceptable solution is the semi-synthetic process, the original concept of which is generally attributed to Potier [30]. Taxotere (docetaxel: Rhone-Poulenc Rorer, France), a semi-synthetic form of taxol, is synthesized from 10-DAB, which is a precursor of taxol and isolated from the needles of European yew, *T. baccata*. The processes of converting 10-DAB and BIII into taxol have been elucidated but at less than 20% yields. Taxotere happens to be a beneficial analogue as a antitumor agent even though taxotere is different at least 2 sites from taxol; it has tert-butoxycarbonyl group instead of the benzoyl

group on the taxol side chain and a hydroxy group instead of acetoyl group on the taxol B ring. Taxotere shows more common side effects such as low WBC counts than taxol, but it was approved by the FDA for use against certain cancers of the breast and ovary. Because taxotere has shown an anticancer activity of 2.5 to 5 times on some tumors [42] and is considered to have another advantage over taxol, viz., solubility in water. It has a shorter infusion time, requires no in-line filter, and has easy administration. On the other hand, Kingston *et al.* (1995, US patent 5,470,866) have reported a new bioconversion method for cephalomannine to taxol, involving steps of dihydroxylation to give cephalomannine-diols, diol cleavage, and benzoylation at the 2-position and reaction with a 1,2-diamine.

Taxol production through cellular cultures. Callus and suspension cultures of *Taxus* sp. have been extensively investigated as an alternative source for taxol production. For efficient initiation and maintenance of plant cell culture, various plant growth hormones and organic substances were supplemented in several basal media such as Gamborg (B5), Murashige and Skoog (MS), SH, TM5, and WPM media (Table 3). The callus and suspension cultures provide several advantages over whole plant cultivation, that is, their ease of establishment and manipulation, lower quantities of pigments, waxy constituents and nonpolar lipids, improvements in efficiency and productivity, rapid growth rate, reducing space requirement, and providing freedom from climatic stress and pests, etc. In addition, cell cultures produce fewer by-products than field plant extracts, which is important for efficient downstream processing and clinical safety. Also, plant cell cultures produce some beneficial analogs which are interesting compounds that have not been previously reported from intact plants, in specific *Taxus* sp. [53]. However, commercial application of plant cell cultures for taxol production is hindered by several limitations. First of all, it requires high inoculation for successful cultivation of *Taxus* sp. Fett-Neto *et al.* [21] reported that typical inocula have to contain approximately one-third of the volume of liquid medium, and Wickremesinhe *et al.* [94] also reported that 80~110 g cell/l based on fresh weight is necessary. In fact, cell growth was drastically reduced when inoculated at densities below 70 g/l, although production of taxol is not related to cell growth. In cell cultures of *T. chinensis*, extracellular and intracellular taxol concentration and ratio were affected by the concentration of inoculum [89]. The concentration of extracellular taxol was high at low inoculum densities, although cell growth and taxol productivity were decreased. However, the taxol excretion ratio of *T. chinensis* was less than 25% in most cases. The mechanism of inoculum size effect on product synthesis may be related to enhanced activities of enzymes in metabolic pathways. Secondly, cell cultures of *Taxus* sp. show very unstable growth and production patterns of taxol, as well as unusually slow growth and low product yields, and cessation of cell growth within 6 to 10 months after initial subcultures [29]. In most cases, taxol concentration fluctuated 100~300 fold during extended cultures in most cell lines [36, 46, 93, 94]. It is also true that plant cell cutures are sensitive to environmental perturbation, subculture routines, initial cell density, and culture temperature. Although, little regional differentiation and less allozyme diversity of *Taxus* sp. was reported [92], severe fluctuation could occur within cultures, individuals, and species. As shown in Table 3, cell culture systems show high fluctuation in taxol production, cell growth, and product secretion according to cell lines used and extended cultures. In particular, high yielding cells produce taxol by more than 60 fold than low-yielding cells even for the same cell line and culture conditions [24]. However, epigenetic stability of high-yielding cell lines is troublesome in most cases, so we need more reliable methods for taxol production. Other problems include fungal contamination, variation of secretion ability, and sharp decrease in taxol concentrations after maximum concentration is reached during the long cultivation time on a commercial scale [57]. Therefore, the establishment of stable and higher-yielding *Taxus* sp. cell lines is necessary for successful taxol production.

For efficient production of taxol, the effect of cultivation environments [23] and various chemicals including precursor, elicitor, and growth retardants, were also reported [24, 82, 98]. The condition of the cell culture is dependent on the cell lines used and the researcher, even though physical factors such as temperature, pH of media, light, agitation speed, and culture scale, are similar: 25~27°C, dark condition, 100~150 rpm in 200 ml media/500 ml flask scale. Recently, overproduction of taxol by gas composition control [57], modifications in sugar type and level [94], precursor feeding [22], elicitor and growth retardant treatment [10, 82], and permeabilization method, as well as choice of auxin and media optimization, show promise for future investigations. Since most of the elicitors and precursors used need organic solvents for solubilization, we have to evaluate the effect of solvent toxicity and taxol productivity. According to Hirasuna *et al.* [36], ethanol exerts a negative effect on taxol and taxane production even in very small doses (0.1% v/v), but appears to have no significant impact on cell growth in *T. baccata*. However, Yukimune [98] successfully used 0.25% (v/v) ethanol for methyl-jasmonate (JA-Me) elicitation in *T. baccata* and *T. x media*, although the timing of addition is an important factor in elicitation. Since Yukimune *et al.* obtained the highest taxol accumulation (153 mg

Table 3. Taxol production in suspension cultures of *Taxus* sp.

Cell lines	Culture type	Medium	Additives	Culture Days	Taxol concentration (secretion ratio)	Reference
T. baccata PC2	Suspension	B5 basal 2% sucrose	5 μM NAA, 0.01 μM BA 2 mM glutamine 50 mg/l ascorbic acid	21	0.15~0.96 mg/l·day (above 90%)	[36]
T. baccata PC2	Suspension (PMB)	B5 basal 2% sucrose	5 μM NAA, 0.01 μM BA 2 mM glutamine 50 mg/l ascorbic acid	30	0.05 mg/l·days (above 90%)	[79]
T. baccata	Suspension	WPM basal 3% sucrose	10 μM NAA	14	0.029 mg/l·day	[98]
T. baccata	Suspension	WPM basal 3% sucrose	10 μM NAA, 100 μM JA-Me	14	3.45 mg/l·day	[98]
T. brevifolia	Suspension	SH basal 6% sucrose	5 mg/l NAA,0.2 mg/l 6-BAP	10	0.143 mg/l·day Max. 1.43 mg/l (N.D)	[47]
T. brevifolia	Suspension	B5 basal 3% sucrose	2.5 mg/l NAA	5	0.4~1.6 mg/l·day (N.D)	Our result
T. brevifolia	Suspension	WPM basal 3% sucrose	10 μM NAA	14	0.014 mg/l·day	[98]
T. baccata	Suspension	WPM basal 3% sucrose	10 μM NAA, 100 μM JA-Me	14	0.036 mg/l·day	[98]
T. canadensis P991	Suspension	B5 basal 2% sucrose	2.7 μM NAA, 0.01 μM BA 0.5 mM ascorbic acid	28	0.21 mg/l·day	[46]
T. canadensis P991	Suspension Wilson type reactor	B5 basal 2% sucrose	2.5 mM NAA, 0.01 mM BA 2 mM glutamine 50 mg/l ascorbic acid	20	1.1 mg/l·day	[67]
T. chinensis	Suspension	MS basal 3% sucrose	0.5 mg/l BA, 0.2 mg/l 2,4-D 0.5 mg/l NAA 100 mg/l ascorbic acid	14	14.1~22.9 μg/l·day (below 25%)	[89]
T. cuspidata P991A2	Suspension	B5 basal 1% sucrose	1 mg/l 2.4-D, 2 g/l CA C_2H_4 5 ppm 10% O_2, 0.5% CO_2 10 g/l fructose (11 days)	25	0.64 mg/l·day Max. 12.2 mg/l (N.D)	[57]
T. cuspidata	Suspension Immobilized	B5 basal 3% sucrose	4 mg/l 2.4-D 1 mg/l kinetin, 15 g/l PVP	180	4.2 μM/l·day (about 66%)	[21]
	Callus			55	0.02%/EDW	
T. cuspidata Sieb & Zucc	Suspension	B5 basal 3% sucrose	4 mg/l 2.4-D 1 mg/l kinetin 15 g/l PVP	44	4 μg/g-EDW	[22]
T. cuspidata Sieb & Zucc	Suspension	B5 basal 3% sucrose	Phenylalanine 0.05 mM or benzoic acid 0.05 mM	40	10 μg/g-EDW	[22]
T. cuspidata	Suspension	B5 basal 2% sucrose	2.7 μM NAA, 0.01 μM BA 2 mM glutamine 0.5 mM ascorbic acid	28	0.53 mg/l·day (14.78 mg/l)	[46]
T. cuspidata Sieb et Zucc	Suspension Immobilized	B5 modify (Nitrogen-limited) 2% sucrose	3 g/l CA, 2 mg/l NAA	40	0.2 mg-excreted/l·day (70~80%)	[75]
T. cuspidata	Suspension Immobilized	B5 modify 2% sucrose	3 g/l CA, 2 mg/l NAA 200 μg/l kinetin	40	0.3 mg/g-DCW·day (N. D)	[76]
Taxus sp. RO1-M28	Suspension	1/4 B5 basal 1% sucrose	10 μM 2,4-D, 2.5 μM zeatin	21	0.28 mg/l·day	[10]

Table 3. Continued.

Cell lines	Culture type	Medium	Additives	Culture Days	Taxol concentration (secretion ratio)	Reference
T. x media cv Hicksii	Suspension	B5 basal 2% sucrose	2 fold B5 vitamin 0.2 g/l CA 0.25% glucose, 0.25% fructose	21	0.283 mg/g-DCW	[94]
T. media	Suspension	WPM basal 3% sucrose	10 μM NAA 10 μM NAA, 100 μM JA-Me	14	2.014 mg/l·day 7.878 mg/l·day	[98]
T. x media Var. Hatfieldii	Callus	WR basal	22.6 μM 2,4-D 100 μM JA-Me	8 weeks	89.98 μg/g-DCW	[26]

Abbreviation: N.D, Not determined; NAA, α-naphthylacetic acid; BAP, benzylaminopurine; PVP, polyvinylpolypyrrolidone; BA, benzyladenine; CA, casamino acid; JA-Me, Methyl jasmonate; EDW, extracted dry weight.

taxol/l) when 100 μM of methyl jasmonate (JA-Me) was added to the medium, the effects of JA-Me were investigated for production of taxanes diterpenes in tissue cultures of many *Taxus* species [26, 58]. Jasmonates are possibly signal compounds in the elicitation process for the biosynthesis of secondary metabolites. According to a recent report [26], the taxol content of *Taxus x media* var. Hatfieldii was increased about 40 fold when added to 100 μM methyl jasmonate in WR media. However, the elicitation effect of JA-Me was not shown in B5 medium, although taxol production from another *Taxus* sp., *Taxus cuspidata*, was elicited by JA-Me. Considering the cell line variability and medium adaptability, the effective elicitator may be different for the researcher and the cell line used.

For continuous production of taxol, excretion is an important factor. *T. baccata*, *T. brevifolia* and *T. cuspidata* may be considered, as they have some active excretion systems for taxol, but *T. x media* shows very low secretion although it has rapid growth characteristics. In our study, we found that taxol excretion was severely affected by the auxin ratio in *T. brevifolia* plant cell culture. Sucrose concentration affected taxol production and cell growth instead of taxol excretion ability. It suggests that the kinds of auxins and ratios in cultures of *Taxus* sp. is a critical factor for taxol excretion and productivity. Also, for efficient continuous production, the production ability has to be maintained stably in all culture periods. However, we found that taxol production showed two peaks in batch cultures of *T. brevfolia*; the first peak at 3~6 days was at a higher concentration than the second peak at 12~18 days. This result coincided with results from cultures of *T. cuspidata* [24]. Unfortunately, no one has given a hypothesis for this phenomenon until now. However, an IPCR (Immobilized Plant Cell Reactor) was successfully used for continuous production of *Taxus cuspidata* and *Taxus brevifolia* by Seki *et al.* [75, 76] and also recently by our group recently. Also, we need more detailed information on the stability and binding affinity of taxol in culture media and extraction solvents. According to Song *et al.* [78], taxol in 1% methanolic aqueous solution has strong nonspecific adsoption for glass, polypropylene, and siliconized polypropylene tubes and the decrease of taxol concentration in plastic tubes was prevented with the addition of 9% fetal bovine serum. These adsorption characteristics of taxol may be used for efficient recovery. For continuous recovery, an extractive reactor or two-phase reactor with immobilization of *Taxus* sp. cells may bring about good results such as higher productivity, preventing hydrolysis of substrate/products, reducing product inhibition, and better integration with chemical steps. But, this needs advanced reactor design and control as well as optimization of operating conditions, because organic solvents introduce the problem of cell toxicity as well as safety and waste disposal issues. Since growth regulators, precursors, and substrates (for BIII in bioconversion etc.) will be extracted during the media recycle, and side products similar to taxol structure and hydrophobicity will be coextracted with taxol, we need a good choice of solvent and fittable partition methods. Nowadays, most researchers in this field recognize that suspension cultures of *Taxus* sp. is the best solution for an economic, reproducible, and rapid production of taxol, and they also believe that vast quantities could be cultured in factory tanks and tapped for the life saving substance. Although it did not contain secretion data, JA-Me treatment on *T. media* showed the best yields (110.3 mg/l over 2 weeks) and the maximum concentration of paclitaxel was reported at 153 mg/l with *T. chinensis* cell cultures, and 10 mg/l·day productivity may be possible in some culture conditions.

Taxol production by fungi. Taxol production by microbial sources provide several advantages over plant cell cultures or direct bark extraction. These include reproducibility and stable productivity, easy adaptation of established simple techniques, production of different bioactive compounds by altering culture conditions, and efficient strain development. Since it was well known that most plants serve as hosts for microbes (which exist

as parasites, saprophytes, endophytes, and symbionts) and because of the genetically transforming ability between host plant and microbe, taxol-producing microorganisms living in *Taxus* sp. and its forest soil were screened intensively. However, it is very difficult and needs high-end techniques because even a single endophytic fungus could produce a lot of antitumor agents and derivatives. To date, three endophytic fungi which could produce taxol and its related diterpene compound, baccatin, have been reported. During a 2-year testing of about 300 fungi isolated from inner bark and needles of yew trees in the USA, using chromatography, mass spectrometry, radiolabelled cultivation, antibody-based immunoassays, and various biological activity assays, only *Taxomyces andreanae* was isolated from a single tree of *T. brevifolia* by Stierle *et al.* [81]. This fungus can produce taxol at 24~50 ng/l and the amount of taxol and baccatin in the fungal extract was estimated at about 1~2 μg/l and 0.5~1 μg/l in a 3-week old culture, respectively. The clue to success may be in the addition of needle broth to the first cultivation media. Since endophytic fungi often cease production of secondary metabolites when removed from the host plant, needle broth may serve as a critical precursor reservoir and genetic promoter for the biosynthetic mechanism of taxol production. On the other hand, this fungus does not produce cephalomanine and taxol or BIII from sodium benzoate-7-C14, Leucine-UL-C14. For this reason, the biosynthesis system of fungi may be different to that of the plant. The hypothesis of intergeneric exchange of the taxol synthesis gene between plant and fungi may need more detailed genetic research although the gene donor has not been decided yet. The gene complex responsible for paclitaxel ring synthesis in *Taxomyces andreanae* was recently isolated by Cytoclonal Pharmaceutics Inc, Texas, in 1996. Recently, *Pestalotiopsis microspora* was isolated from the Himalayan Yew, *Taxus wallichina* by Strovel *et al.* [84]. This wild-type strain produced 60~70 μg/l taxol in 3 weeks although the taxol content sharply decreased after the 3 week cultivation. This is more than 1,000 times the taxol production from *Taxomyces andreana*. If this strain is used in genetic engineering or metabolic engineering, economic mass production could easily come true. Later, an unidentified taxol producing white fungi was reported by Qiu *et al.* [69] from *Taxus yunnanensis*. These types of fungi will be useful as gene pool resources for genetic engineering, as well as alternative resources of taxol. Also, they can provide a reasonable model system to elucidate the taxol resistance mechanism and avoidance of suicide.

Prospects and Commercial Feasibility

It is true that the clinical cost of taxol is higher than that of standard chemotheraphy. Currently, the retail price of taxol is $1,900 for seven 30 mg vials and the wholesale price is supposed to be $4.87/mg (the market for taxol from the yew tree was $660 million in 1995 and is projected to exceed one billion in 1997). But, the price of this drug may drop seriously after this year. As Bristol-Meyers Squibb Co.'s exclusivity on taxol expired in December 1997, Hauser Chemical Research Inc. (HCRI) and American Home Products (AHP) prepared to sell in the USA and world-wide. HCRI has a 200 kg/yr taxol production plant, but they produced only 10~12 kg taxol/yr during the last 2 years with a sharp drop of net income. The reason was that BMS, the biggest customer of HCRI, did not renew their contract after the first 3-year $100 million contract in 1991. As most companies, including BMS and Rhone-Poulenc Rorer, wanted to buy and sell taxol produced from a semisynthetic source such as Taxotere (docetaxel), instead of natural sources, a more stable and cheaper supply would be possible. Also, large-scale production of taxane by plant cell culture is already being attempted by Phyton Catalytic (Ithaca, NY), SamYang Genex (Taejeon, Korea), and Mitsui Petrochemicals Industries (Yamaguchi, Japan). Escagentics Inc. (1993), Penn State Research Foundation (1993), Nippon Steel Corp. (1992), and the US Department of Agriculture (1991) among others, have equipment for taxol production and patents for taxane manufacturing methods. Recently, Union Chemical Laboratory in the USA succeeded in taxol hyperproduction with a Taiwanese yew plant culture using a 5 l reactor for 30 days. They produced 40 mg taxol/l and showed above 95% purity following a simple downstream process (ITRI Today No. 6, Fall, 1996. Union Chemical Laboratory). Soon after, Cytoclonal Pharmaceutics Inc. (CPI) wanted to produce taxol by fungal fermentation. In 1996, CPI obtained the rights to a patented fungus and developed a large-scale production system for taxol. Also, Paxetol, which is a brand of paclitaxel for injection, is being prepared for marketing via strategic manufacturing, distribution, sales, and alliances in domestic and international markets.

The severe drawback of taxol during actual application, of low solubility in aqueous solution, will be circumvented by new DDS and modification of taxol, although this has not yet been approved by the FDA. Solubility of taxol was increased 1,600 times by acyl group attachment using a microbial lipase or thermolysin. This type of taxol will contribute to the decrease of production cost via longer retention time in the body, and minimization of dosage and side effects. In the future, taxol should lose its notorious position of high cost due to the development of economical plant cell cultures and fungal or bacterial fermentation, as well as efficient recovery and advanced DDS.

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