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Taxane Anticancer Agents

Basic Science and Current Status

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Chapter 6

Bioactive Metabolites of the Endophytic Fungi of Pacific Yew, *Taxus brevifolia*

Paclitaxel, Taxanes, and Other Bioactive Compounds

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The endophytic microbes associated with the Pacific yew tree, *Taxus brevifolia*, were examined as potential alternative sources of the anticancer drug taxol, a secondary metabolite of the host organism. A novel fungus, *Taxomyces andreanae*, isolated from the inner bark of a yew tree growing in northwestern Montana, appears to produce taxol and other taxanes, in *de novo* fashion when grown in semi-synthetic liquid media. The presence of taxol in the fungal extract was confirmed by mass spectrometry, comparative chromatographic behavior with yew taxol, reactivity with taxol-specific monoclonal antibodies, and 9KB cytotoxicity studies. Both acetate-1-^{14}C and phenylalanine UL-^{14}C served as precursors of taxol-^{14}C in fungal culture labeling studies, confirming the *de novo* synthesis of taxol by the fungus. Immunoassay techniques are currently being used to screen extracts of *Taxomyces andreanae* for new taxanes, and to determine if other endophytic fungi are taxol producers. Fungal endophytes used in this study are further screened for additional biological activity following taxol/taxane analysis.

Background

Cancer is the second leading cause of death in the United States, and the incidence of cancer continues to climb annually (*1*). Chemotherapeutic agents are instrumental in the fight against this dreaded disease, and effective anticancer agents, particularly those that affect refractory tumors, are critical objectives in western medicine. Taxol (**1**) is a new anticancer drug with particular efficacy against refractory breast and ovarian cancers (*2, 3*). Although initially isolated and characterized in 1971, taxol did not achieve notoriety until 1977, when its strong activity against human tumor xenograph

NOTE: Paclitaxel is the generic name for Taxol, which is now a registered trademark.

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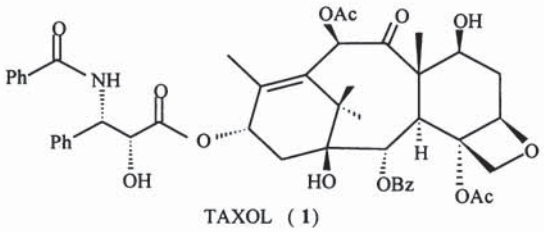
systems and murine B16 melanoma cells prompted its development towards clinical trials (*4*). Interest in taxol intensified when its unique mode of antimicrotubule cytotoxicity was published in 1979 (*5, 6*). Microtubules are among the most strategic of the subcellular chemotherapeutic targets. Antimicrotubule agents are extremely potent, requiring only a few molecules to disrupt the microtubular structure of cancer cells (*2*). These compounds, which include the vinca alkaloids, are among the most important anticancer drugs currently employed (*2*).



TAXOL (1)

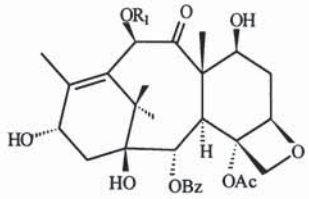
Taxol: the Supply Dilemma. But there is a problem with taxol. This highly functionalized diterpene is isolated primarily from the inner bark of the relatively rare and slow growing Pacific yew tree, *Taxus brevifolia*, and a few related species, in extremely small yields (< .02% dry weight) (*7*). The emergence of taxol as an effective anticancer agent created a dilemma: how to insure an adequate supply of a compound of non-microbial origin. Athough the pharmaceutical potential of taxol elevated the status of the yew tree from a nuisance weed to a precious commodity and natural resource, it did not alter the underlying dilemma - there were simply not enough yew trees to supply the growing demands for taxol (*4*). Advanced preclinical and phase I clinical development of taxol required several collections ranging in size from 5,000 to 15,000 pounds of dry bark. A mature Pacific yew (100 years old) yields approximately 10 lb. of dry bark, so each collection required the sacrifice of 500 to 1500 trees (*4*). As the efficacy of the compound became more apparent, the demand for additional taxol increased (*4*).

In 1987-1988 a 60,000 lb bark collection was undertaken with little controversy. The need for a second 60,000 lb bark collection in 1989, however, sparked concern about the impact such collection sizes may have on the continued existence of the yew tree. Although no accurate inventories of the tree have ever been undertaken, the Fish and Wildlife Service of the U.S. Department of the Interior states that "Much of the range of the yew has not been subject to statistical inventories, especially the northern portion (i.e., Alaska and British Columbia). Nonetheless, based on stand information, together with satellite imagery, the U.S. Forest Service estimates that 130 million yew trees occur on 1,778,000 acres of National Forest in the Washington and Oregon Cascades, and Oregon Coast Range" (*4*).

Even with this estimate, however, it is clear that additional sources of taxol must be found. A single course of clinical treatment is 125-300 mg of taxol, and typical treatments may extend for 10 or more courses. Treatment of the 12,000 women who die annually of ovarian cancer alone would consume as much as 36 kg of the drug (*4*). With current isolation methodologies, 1 kg of taxol is isolated from 25,000 lb of dried bark, or the bark of 2500 yew trees. Therefore, simply treating ovarian cancer over a one year period would consume 90,000 mature yew trees (*4*). The recent approval of

taxol for treatment of breast cancer will triple the projected demand. Should taxol prove as effective against other refractory cancers, including head and neck and non-small cell lung cancers as clinical trials have indicated (*8, 9*,), it is not unreasonable to assume that the demand for taxol may exceed 300 kg, or 750,000 trees per year (*4*). This represents an enormous demand on a limited resource. There are simply not enough yew trees growing in North America to satisfy projected needs of this drug over the next twenty years (*4*).

Several research groups have labored to alleviate the supply problem using a variety of strategies. Total syntheses from simple precurors were published virtually simultaneously by Robert Holton (*10*) and K.C. Nicolaou (*11*), and Paul Wender's elegant approach to total synthesis will probably be realized before this symposium is published (*12*). Although these synthetic efforts are chemical masterpieces, they will not answer the supply question. Semisynthetic methods using taxoid starting materials have proven successful, however, and will facilitate taxol availability. Most of these syntheses begin with either baccatin III or 10-deacetylbaccatin III which can be isolated from the needles of the European yew *Taxus baccata*. Yew needles are a renewable resource and should provide an adequate supply of the necessary starting materials. Several semisynthetic methods have been proposed (*13, 14*), but the most promising utilize the strategies devised by Holton (*15*), Georg (16) and Ojima (*16*). Plant tissue culture also shows promise, and some research groups are reporting yields commensurate with commercialization (*17*).



BACCATIN III R_1= Ac
10-DEACETYLBACCATIN III R_1 = H

Microbial Source for Taxol

Our own attempt at easing the supply dilemma focused on the discovery of a new biological source of the drug: an endophytic microbe colonizing the yew tree. Over the last two years we have isolated over 300 fungi from the bark and needles of yew trees in Montana, Washington, Idaho, and Oregon. Promising taxol producers have been studied using a variety of different techniques, including chromatography, mass spectrometry and antibody based immunoassays. Immunoassay is proving an effective tool not only in assessing the presence of taxol and taxanes in crude extracts, but also in providing an efficient fractionation guide.

We were painfully aware from project inception that our chances of success were pretty minimal. In an effort to justify the tremendous time expenditure of this venture we broadened our research goals. Microbial extracts were evaluated not only for evidence of taxoids but also for other bioactive components. Particular attention was paid to compounds with either antifungal or anticancer potential. Fungi already provide a number of important antibiotics, including the penicillins and cephalosporins

(*18*). Endophytic fungi, however, particularly those isolated from conifers, are an untapped reservoir of compounds with pharmaceutical potential. Fungi associated with medicinal plants might prove a good source of novel bioactive compounds, and a taxol-producing fungus would be a noteworthy beginning.

Advantages of Microbial Source. From a practical viewpoint, microbial fermentation as a means of producing bioactive substances has several advantages (*19*).

1. Industrial production of a bioactive substance like taxol requires reproducible, dependable productivity. If a microbe is the source organism, it can be grown in tank fermentors as needed, producing a virtually inexhaustible supply of taxol (*19*).
2. Microorganisms typically respond favorably to routine culture techniques. Cultivation of macroorganisms (tissue culture) is considerably more challenging, requiring either specialized techniques or months of growth before harvesting is feasible (*19*).
3. Productivity amplification is relatively easy in microorganisms. In the case of penicillin, improved culture conditions and genetic manipulation of producing strains of *Penicillium* increased drug yield from a few micrograms per milliliter to thousands of micrograms per milliliter (*19, 20*). With macroorganisms, larger collection sizes are the most reasonable option for improved productivity. In the case of taxol, larger collection sizes will lead to the eradication of the source organism within a few years if all of the demands for it are to be met.
4. Different bioactive compounds can be produced by altering culture conditions. The antibiotic aplasmomycins were produced by *Streptomyces griseus SS-20* only after NaCl was added to the medium (*21*). Directed changes in culture conditions can be explored indefinitely as a method of optimizing various biosynthetic pathways, that may lead to even more effective derivatives of taxol (*19*).

What all of this means is that a **microbial source of taxol could provide an inexhaustible supply of taxol and novel taxanes**.

The Gibberellins: Precedence for Taxol-Producing Microbe? The search for a taxol producing fungus was prompted by the advantages inherent in a microbial drug source. The real motivation for this search, however, was a discovery made forty years earlier by Yabuta, in his study of "foolish rice seedling disease". He determined that the gibberellins, also highly functionalized diterpenes, were responsible for the disease symptoms induced by the phytopathogenic fungus *Gibberella fujikuroi* (*22*). It has since been established that the gibberellins are ubiquitous phytohormones produced by most higher plants. The pathways of gibberellin biosynthesis in the fungus and the higher plant are identical up to gibberellic acid-12 (*23*). This suggests the possibility of intergeneric-genetic exchange between higher plant and fungus. This type of exchange would probably require an intimate association between the cells of the tree and its microbial associates. Therefore, a search for a taxol producing microorganism should (and did) commence in the tissues of *Taxus spp*., particularly in the portions of the tree in which taxol is isolated (*7*).

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