Antifungal properties of taxol and various analogues

D. H. Young[a], E. L. Michelotti[a], C. S. Swindell[b] and N. E. Krauss[b]

[a]*Rohm and Haas Co., Research Laboratories, Spring House (Pennsylvania 19477, USA), and* [b]*Department of Chemistry, Bryn Mawr College, Bryn Mawr (Pennsylvania 19010, USA)*
Received 7 April 1992; accepted 23 April 1992

Abstract. The antimitotic agent taxol was tested for toxicity towards fungi from different taxonomic groups and found to be particularly active against oomycete fungi. In germinating zoospore cysts of the oomycete *Phytophthora capsici* the mechanism of action of taxol was shown to involve inhibition of mitosis, presumably resulting from an effect on microtubules. Various taxol analogues with deleted A-ring C-13 side chain substituents were tested for toxicity towards *P. capsici* and *Aphanomyces cochlioides* to provide insight into structural features required for activity. The importance of the side chain was shown by the much lower activity as compared to taxol of analogues lacking all or part of the side chain. The effect of stereochemistry at the C-2′ position on fungitoxicity towards oomycetes was similar to that reported previously on mammalian microtubule assembly.
Key words. Taxol; antifungal; *Phytophthora capsici*; nuclear division; microtubules.

Taxol[1], an alkaloid isolated from the Pacific yew (*Taxus brevifolia*), is an experimental anticancer drug which binds to microtubules and inhibits mitosis by preventing the normal formation of the mitotic spindle[2]. In contrast to other antimicrotubule agents which prevent nuclear division by promoting disassembly of microtubules, taxol stabilizes microtubules against depolymerization both in vivo[3] and in vitro[4]. It lowers the critical concentration of tubulin required for microtubule assembly, and induces assembly under otherwise unfavorable conditions including low temperature, the absence of microtubule-associated proteins or GTP, and the presence of calcium ions[2].

Taxol affects a wide variety of eukaryotic cells including mammalian cells[3,5], *Xenopus*[6] and sea urchin eggs[7], *Haemanthus* endosperm[8], *Poterioochromonas*[9], and *Trypanosoma*[10]. However, to our knowledge reports of activity against fungi are limited to the slime mould *Physarum polycephalum* and the bean rust fungus *Uromyces phaseoli*. In *P. polycephalum*, taxol blocks mitosis in amoebae at 10 μM[11], and inhibits cold-induced disassembly of microtubules in vitro[12]. In *U. phaseoli*[13], partial stabilization of microtubules against cold depolymerization has been reported, but only at a high taxol concentration (100 μM).

In this study we examine the fungitoxicity of taxol towards various fungi from different taxonomic groups, its effects on nuclear division in the oomycete *Phytophthora capsici*, and report structure-activity information for various taxol analogues.

Materials and methods

Phytophthora capsici (ATCC 15399), *Aphanomyces cochlioides* (ATCC 38470), *Pythium splendens* (ATCC 14557), *Pythium ultimum* (ATCC 26083), *Saprolegnia ferax* (ATCC 36052) and *Aspergillus nidulans* (ATCC 36321) were from the American Type Culture Collection, Rockville, Maryland. All other fungi were from the culture collection of the Rohm and Haas Exploratory Agricultural Products Research Department. Organisms were maintained on potato dextrose agar at 25 °C. For production of zoospores, *P. capsici* was grown on V-8 agar under conditions which promote sporulation, and the zoospores were harvested after incubation at 4 °C to induce zoospore release[14].

Poison agar assays were performed by adding test compounds dissolved in dimethylsulfoxide (12.5 μl) to 2.5-ml aliquots of potato dextrose agar (PDA) at 50 °C, then pouring the molten agar immediately into 6-well tissue culture plates (well diameter 35 mm). After the agar had hardened, the plates were inoculated in the center with 7-mm diameter plugs taken from the growing edges of fungal cultures. In the case of *Aspergillus nidulans*, plates used for inoculum were prepared by spreading a spore suspension on PDA and incubating the plates for 3 days at 25 °C. The increase in colony diameter was measured after incubation at 25 °C for 20–120 h depending on the growth rate of the particular organism, and EC_{50} values were computed from median effect plots[15].

To examine the effect of taxol on nuclear division in germinating zoospore cysts of *Phytophthora capsici*, wells of Lab-Tek 2-chamber slides (Miles Scientific, Naperville, JL) were supplied with 0.4 ml of liquid asparagine-sucrose medium[16] containing the appropriate concentration of taxol followed by 0.4 ml of freshly prepared zoospore suspension containing 2×10^5 zoospores/ml. This provided a thin layer of liquid which encouraged zoospore encystment and attachment to the slide. After incubation at 25 °C for the appropriate period, slides were fixed in glutaraldehyde, stained with mithramycin and examined by epifluorescence microscopy[14]. The number of nuclei per cell was determined by examining 200 cells per treatment. To measure the effect of taxol on germ tube elongation, chamber slides were fixed after various treatment times and the mean length of germ tubes was calculated from measurements of 50 germlings per treatment taken from photographic enlargements. In experiments to evaluate the

speed of effect of taxol on nuclear division, zoospores were incubated in chamber slides until approximately the first nuclear division (1.5 h). The medium was then replaced with twofold diluted medium containing taxol, and slides were fixed at intervals thereafter for subsequent mithramycin staining. In poison agar assays designed to compare the effect of taxol on mycelial growth with its effect on nuclear division, taxol was incorporated in twofold diluted asparagine-sucrose medium containing 0.8% agar, the medium inoculated with mycelial plugs, and colony diameter measured after incubation for 48 h at 25 °C.

The synthesis and characterization of the taxol analogues used in this study have been reported previously[17]. Taxol was kindly provided by Dr M. Suffness of the National Cancer Institute.

Results and discussion

All oomycete fungi tested were highly sensitive to taxol, whereas fungi from other taxonomic groups were relatively insensitive (EC_{50} > 50 μM) with the exception of the basidiomycete *Stereum purpureum* (table 1). EC_{50} values for growth inhibition of oomycetes by taxol ranged from 0.4 to 5.9 μM, and were only slightly higher than corresponding values reported in the literature for inhibition of various mammalian cell lines (0.32 μM for murine P388 leukemic cells[18], 0.35 μM for chinese hamster ovary cells[17], and 0.09 μM for the murine macrophage-like cell line J774.2[17]).

Table 1. Toxicity of taxol towards fungi from different taxonomic groups

Organism	Taxonomic group	EC_{50} (μM)
Aphanomyces cochlioides	Oomycetes	0.4
Pythium splendens	Oomycetes	1.1
Pythium ultimum	Oomycetes	1.4
Phytophthora capsici	Oomycetes	1.6
Saprolegnia ferax	Oomycetes	5.9
Stereum purpureum	Basidiomycetes	9.6
Lentinus lepideus	Basidiomycetes	> 50
Piricularia oryzae	Deuteromycetes	> 50
Fusarium roseum	Deuteromycetes	> 50
Botrytis cinerea	Deuteromycetes	> 50
Monilinia fructicola	Ascomycetes	> 50
Diaporthe sojae	Ascomycetes	> 50
Ceratocystis ulmi	Ascomycetes	> 50
Aspergillus nidulans	Ascomycetes	> 50

Table 2. Effect of taxol on germ tube length as a function of time in *Phytophthora capsici*

Treatment time (h)	Germ tube length (μm) Control	Taxol (8 μM)
2	45.3 ± 4.1	51.3 ± 4.3
3	61.6 ± 9.7	60.2 ± 7.1
4	79.6 ± 7.4	77.9 ± 7.7
5	130.5 ± 10.5	99.7 ± 8.6*
6	196.3 ± 10.2	124.6 ± 8.9*

Data represent mean values ± standard error. Asterisks indicate significant differences between taxol-treated and untreated control cells (t test, p = 0.05).

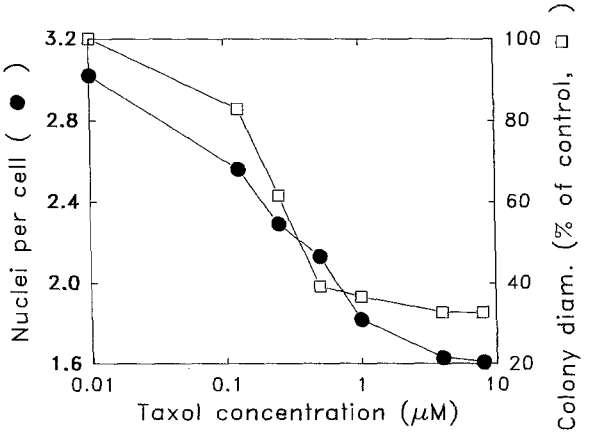


Figure 1. Effect of taxol on the number of nuclei per cell in *P. capsici* germlings 3 h after treatment of zoospores, and on radial growth in poison agar assays. In untreated control cells the mean number of nuclei per cell was 3.02.

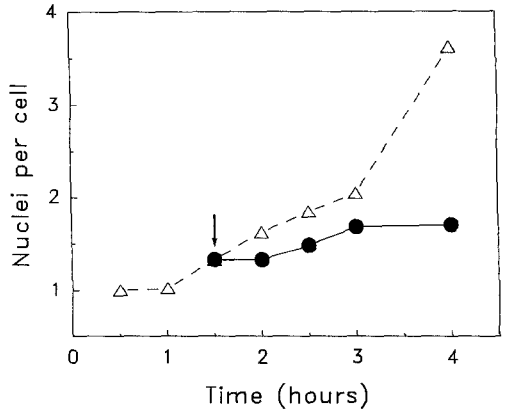
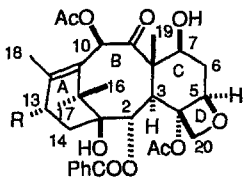
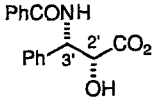


Figure 2. Speed of action of taxol (8 μM) in inhibiting nuclear division in *P. capsici* germlings. Taxol was added at 1.5 h (arrow), and the number of nuclei per cell in taxol (●) and control treatments (△) was followed over time.

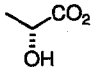
The effect of taxol on nuclear division in *P. capsici* was studied in order to evaluate whether its growth-inhibitory effects on oomycete fungi result from inhibition of mitosis, as is the case in other organisms[2, 8, 9, 11]. Encystment of zoospores and germination were unaffected by 8 μM taxol in the chamber slide test system (data not shown), and germ tube elongation was not inhibited until after 4 h (table 2). In contrast, mithramycin staining showed that nuclear division was strongly inhibited within 3 h by taxol at growth-inhibitory concentrations of 0.125–8 μM (fig. 1). Taxol also inhibited nuclear division very rapidly when added to germlings undergoing the first cycle of nuclear division (fig. 2).

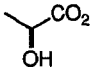
Since inhibition of nuclear division by taxol was extremely rapid, preceded the effect on cell growth, and occurred in the same concentration range as the effect on growth, inhibition of mitosis does appear to be involved in the mechanism of action. Presumably, this results from the interaction of taxol with microtubules as has been established in mammalian cells[2].

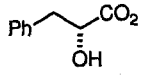


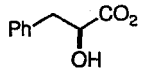
1 R =  Taxol

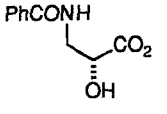
2 R = HO Baccatin III

3 R =  Baccatin III 13-(R-lactate)

4 R =  Baccatin III 13-(S-lactate)

5 R =  Baccatin III 13-(R-3-phenyllactate)

6 R =  Baccatin III 13-(S-3-phenyllactate)

7 R =  Baccatin III 13-(R-N-benzoyl-iso-serinate)

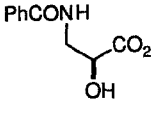
8 R =  Baccatin III 13-(S-N-benzoyl-iso-serinate)

Figure 3. Structures of taxol analogues.

The structures of taxol (1), baccatin III (2), and the taxol deletion analogues 3–8 investigated in the present study are given in figure 3. The ability of these substances to promote assembly of calf brain tubulin into microtubules has been investigated previously[17,19]. In the assembly assay 5–7 were the more active of the analogues, but were not as active as taxol, 3 and 4 were less active, while baccatin III and 8 were essentially inactive. The cytotoxicities of these compounds towards mammalian cell lines paralleled their calf brain microtubule assembly activities[17,19].

The toxicities of the taxol analogues towards the oomycetes *A. cochlioides* and *P. capsici* are summarized in table 3. Taxol was clearly the most toxic compound towards both organisms indicating that as in other cell systems[12,17,19] the A-ring C-13 side chain is important for activity. Baccatin III and analogues 5 and 6 exhibited intermediate toxicity towards *A. cochlioides*; baccatin III and analogues 7 and 8 exhibited intermediate toxicity towards *P. capsici*. Since the isolation and in vitro assembly of oomycete tubulin has not yet been achieved, a comparison of the cytotoxicity of taxol, baccatin III, and taxol analogues 3–8 with their effect on microtubule assembly is not possible at present. It is tempting to draw conclusions about the relative abilities of these compounds to interact with oomycete microtubules from their cytotoxicities towards *A. cochlioides* and *P. capsici*, since a parallel exists between these properties in corresponding mammalian systems. However, Lataste et al.[12] have shown that although taxol and taxanes like baccatin III which lack the taxol C-13 side chain inhibit the disassembly of *Physarum* microtubules in vitro, only taxol is cytotoxic towards *Physarum* and *Plasmodium* cultures. Evidently, the taxol C-13 side chain is crucial for the in vivo accessibility of drug to the amoebal microtubules.

It is probably safer to draw conclusions for epimeric pairs, i.e., compounds 5/6 and 7/8, since their respective structural similarities suggest that each pair should possess similar bioavailability. For the 5/6 pair, the greater sensitivity of *A. cochlioides* towards the C-2′ *S* stereoisomer (compound 6) recalls the behavior of this pair of epimers in the calf brain microtubule assembly assay[17], although it is exaggerated with *A. cochlioides*. Likewise for the 7/8 pair, the greater sensitivity of both *A. cochlioides* and *Phytophthora capsici* towards the C-2′ *R* stereoisomer (compound 7) is similar to the response of mammalian microtubules towards these compounds[17]. The differential sensitivity of *A. cochlioides* and *P. capsici* towards compounds 5–8 is intriguing, however.

Lataste et al.[12] have suggested that the ability of taxol to inhibit the disassembly of both amoebal and mammalian microtubules implicates a highly conserved taxol binding region on tubulin. Once technically feasible, it would be worthwhile to characterize the in vitro effects of taxol, baccatin III, and analogues 3–8 on assembly of oomycete microtubules. This endeavour would allow a stricter comparison of the ligand structural requirements of the oomycete and mammalian microtubular taxol binding regions.

Table 3. Toxicity of taxol analogues towards *A. cochlioides* and *P. capsici*

Compound	EC_{50} (μM) *A. cochlioides*	*P. capsici*
1	0.4	1.6
2	60.6	26.5
3	121.6	150.4
4	197.8	244.5
5	62.4	>250
6	10.7	>250
7	119.5	11.7
8	208.1	48.1

Acknowledgment. Partial support through USPHS Grant No. 41349 (to C.S.S.) awarded by the National Cancer Institute, DHHS is gratefully acknowledged. We thank A. Egan for critical reading of the manuscript.

1 Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P., and McPhail, A. T., J. Am. chem. Soc. *93* (1971) 2325.
2 Manfredi, J. J., and Horwitz, S. B., Pharmac. Ther. *25* (1984) 83.
3 Schiff, P. B., and Horwitz, S. B., Proc. natl Acad. Sci. USA *77* (1980) 1561.
4 Schiff, P. B., Fant, J., and Horwitz, S. B., Nature *277* (1979) 665.
5 De Brabander, M., Geuens, G., Nuydens, R., Willebrords, R., and De Mey, J., Proc. natl Acad. Sci. USA *78* (1981) 5608.
6 Heidemann, S. R., and Gallas, P. T., Devl Biol. *80* (1980) 489.
7 Schatten, G., Schatten, H., Bestor, T. H., and Balczon, R., J. Cell Biol. *94* (1982) 455.
8 Bajer, A. S., Cypher, C., Molè-Bajer, J., and Howard, H. M., Proc. natl Acad. Sci. USA *79* (1982) 6569.
9 Herth, W., Protoplasma *115* (1983) 228.
10 Baum, S. G., Wittner, M., Nadler, J. P., Horwitz, S. B., Dennis, J. E., Schiff, P. B., and Tanowitz, H. B., Proc. natl Acad. Sci. USA *78* (1981) 4571.
11 Wright, M., Moisand, A., and Oustrin, M. L., Protoplasma *113* (1982) 44.
12 Lataste, H., Senilh, V., Wright, M., Guénard, D., and Potier, P., Proc. natl Acad. Sci. USA *81* (1984) 4090.
13 Hoch, H. C., and Staples, R. C., Protoplasma *124* (1985) 112.
14 Young, D. H., Pestic. Biochem. Physiol. *40* (1991) 149.
15 Chou, T., J. theor. Biol. *59* (1976) 253.
16 Erwin, D. C., and Katznelson, K., Can. J. Microbiol. *7* (1961) 15.
17 Swindell, C. S., Krauss, N. E., Horwitz, S. B., and Ringel, I., J. med. Chem. *34* (1991) 1176.
18 Guéritte-Voegelein, F., Guénard, D., Lavelle, F., Le Goff, M., Mangatal, L., and Potier, P., J. med. Chem. *34* (1991) 992.
19 Parness, J., Kingston, D. G. I., Powell, R. G., Harracksingh, C., and Horwitz, S. B., Biochem. biophys. Res. Commun. *105* (1982) 1082.

0014-4754/92/090882-04$1.50 + 0.20/0

Anti-muscarinic activity of a family of $C_{11}N_5$ compounds isolated from *Agelas* sponges

R. Rosa[a], W. Silva[b], G. Escalona de Motta[c], A. D. Rodríguez[a], J. J. Morales[a] and M. Ortiz[a]

[a]*Department of Chemistry, University of Puerto Rico,* [b]*Department of Pharmacology, Universidad Central del Caribe, School of Medicine, and* [c]*Institute of Neurobiology, Medical Sciences Campus, University of Puerto Rico, Blvd del Valle 201, San Juan (Puerto Rico 00901, USA)*
Received 30 December 1991; accepted 27 April 1992

Abstract. In a search for potential target sites for $C_{11}N_5$ compounds obtained from marine sponges of the genus *Agelas* we evaluated their interaction with muscarinic acetylcholine receptors from rat brain membranes. In competition experiments with ^{3}H-QNB these compounds displayed the following rank order of potency: sceptrin > oroidin ≥ dibromosceptrin ≥ clathrodin. Sceptrin (50 μM) was shown to be a competitive inhibitor of ^{3}H-QNB binding as revealed by Scatchard analysis. The results demonstrate the ability of these compounds to interact with multiple target molecules in the micromolar range.
Key words. Marine sponges; muscarinic receptor; sceptrin; oroidin; dibromosceptrin; clathrodin.

Marine organisms are good sources of compounds that act on specific sites of cell membranes. For example marine neurotoxins such as the dinoflagellate saxitoxin and the sea anemone toxin II are very selective pharmacological probes[1–5]. In our laboratory we are examining a variety of marine organisms from around Puerto Rico in an effort to identify new substances with specific biological activities.

Sponges of the genus *Agelas*, collected off-shore western Puerto Rico near Desecheo Island, were extracted in methanol and separated chromatographically. The chemical structures of compounds present in the purified fractions were elucidated using spectroscopic methods. A family of $C_{11}N_5$ compounds with different bromine substitutions in the pyrrole ring were identified: clathrodin (**1**), the only nonbrominated compound of the family, oroidin (**3**), the dibrominated analog of clathrodin, sceptrin (**4**), the 2 + 2 cycloaddition product dimer of hymenidin (**2**), and dibromosceptrin (**5**), the corresponding dimer of oroidin (fig. 1)[6–10]. Recently, Kobayashi et al. showed that some members of this family have serotonergic and adrenergic antagonist activity, while oxysceptrin is an actomyosin ATPase activator[11–13]. In this study we have specifically assessed the ability of these $C_{11}N_5$ compounds to interact with muscarinic acetylcholine receptors (mAChR) in rat brain membranes via radio receptor binding assays.

Materials and methods

Extraction and purification of alkaloids from Agelas sponges. The extraction and purification of clathrodin (**1**) was performed as described by Morales and Rodríguez[7]. The sponge was stored at 0 °C prior to freezing and lyophilization. The methanol (MeOH) extract of the sponge was suspended in H_2O and extracted succesively with chloroform ($CHCl_3$ (3 × 250 ml)) and normal butanol (n-BuOH (2 × 250 ml)). After concentration in vacuo the n-BuOH soluble portion (3.30 g) was chromatographed on a reversed phase (C_{18}, 20 g) column with water (H_2O) followed by a silica (Si) gel column (48 g) with $CHCl_3$-MeOH (4 : 1) saturated with ammonia (NH_3). Combination of like fractions on the basis of thin layer chromatography (TLC) analyses gave pure clathrodin (**1**) as a colorless semisolid (840 mg).

The extraction and purification of sceptrin (**4**) and oroidin (**3**) was performed as follows: *Agelas conifera* was