Covalent Binding of the Benzamide RH-4032 to Tubulin in Suspension-Cultured Tobacco Cells and Its Application in a Cell-Based Competitive-Binding Assay

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The benzamide, RH-4032, was found to be a potent antimicrotubule agent in tobacco (*Nicotiana tabacum*) cells. It strongly inhibited root growth and produced swollen club-shaped roots, an accumulation of cells in arrested metaphase, and loss of microtubules. RH-4032 inhibited the in vitro assembly of bovine tubulin into microtubules, with inhibition requiring a relatively long incubation period. Treatment of tobacco suspension-cultured cells or isolated bovine tubulin with [^{14}C]RH-4032, and analysis of radiolabeled protein revealed a highly specific covalent attachment to β-tubulin. Binding of [^{3}H]RH-4032 in tobacco suspension-cultured cells was shown to be saturable and to be influenced by pre-incubation of the cells with various antimicrotubule agents: Binding of [^{3}H]RH-4032 was inhibited by the benzamides, pronamide and zarilamide, the *N*-phenylcarbamate, chlorpropham, and the microtubule-stabilizing drug, paclitaxel, whereas trifluralin and amiprophosmethyl were not inhibitory. A common characteristic of agents that cause microtubule disassembly was a slight enhancement of [^{3}H]RH-4032 binding at low concentrations, which did not occur with the microtubule-stabilizing agent paclitaxel. For structural analogs of RH-4032 and various *N*-phenylcarbamates, it was shown that the ability to inhibit binding of [^{3}H]RH-4032 was correlated with the ability to inhibit tobacco root elongation. The results suggest a common binding site on β-tubulin for RH-4032, pronamide, zarilamide, and chlorpropham, which is distinct from the binding site(s) for trifluralin and amiprophosmethyl. RH-4032 provides a unique approach to studying effects of antimicrotubule agents on plant cells by allowing competitive tubulin binding assays to be conducted in whole cells.

Tubulin is the biochemical target of a wide variety of drugs and pesticides that inhibit nuclear division by disruption of microtubule function (Morejohn and Foskett, 1986; Hamel, 1996). Agents that affect microtubules in plant cells include commercial herbicides such as the dinitroaniline class, the *N*-phenylcarbamate class, the benzamide, pronamide, the phosphoric amide, amiprophosmethyl (Morejohn and Foskett, 1986; Akashi et al., 1988), as well as the antifungal, benzamide zarilamide (Young, 1991), and the anticancer drug, paclitaxel (Morejohn and Foskett, 1986). RH-4032 is one analog from a new class of antitubulin benzamides that inhibit the growth of fungi, plants, mammalian cells, algae, and protozoans. RH-4032 is particularly active toward plant cells whereas a close analog, RH-7281, demonstrates sufficient selective toxicity toward Oomycete fungi as compared with plants to be useful as an agricultural fungicide (Egan et al., 1998). This report describes studies on the mechanism of action of RH-4032 and its unusual ability to bind covalently in a highly specific manner to tubulin when applied to whole cells. This property has enabled the development of a unique binding assay in tobacco (*Nicotiana tabacum*) cells, which can be used to study effects of antitubulin compounds in a whole cell system using radiolabeled RH-4032 as a probe.

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RESULTS

Effects on Root Growth, Mitosis, and the Microtubule Cytoskeleton

RH-4032 (Fig. 1) did not prevent the germination of tobacco seeds, but strongly inhibited subsequent root growth. The emergent root tips were swollen and club-shaped (Fig. 2B), morphological effects that are characteristic of compounds that arrest mitosis by affecting microtubules (Vaughn and Lehnen, 1991). As shown in Figure 3, RH-4032 was much more potent than the structurally related benzamides, pronamide and zarilamide, which are known to act on microtubules (Akashi et al., 1988; Young, 1991), and it was also more active than other antimicrotubule herbicides (the phosphoric amide, amiprophos-methyl, the dinitroaniline, trifluralin, and the *N*-phenylcarbamate, chlorpropham).

Further evidence for inhibition of mitosis by RH-4032 was obtained by cytological analysis of root tip and suspension-cultured cells. In root tip cells RH-4032 caused an accumulation in arrested metaphase of cells containing scattered, paired chromosomes that failed to align at the metaphase plate (Fig. 2D). A similar time-dependent accumulation of cells in arrested metaphase was found using suspension-cultured cells (Table I). To determine whether inhibition of mitosis was accompanied by microtubule disruption, immunofluorescent staining was used to visualize cellular microtubules in suspension-cultured cells. Arrays of cortical and mitotic spindle microtu-

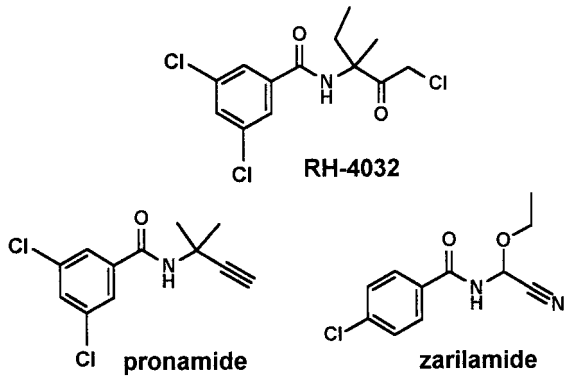


Figure 1. Structures of the benzamides RH-4032, pronamide, and zarilamide.

bules were observed in untreated control cells (Fig. 2, E and F), but were absent in cells treated with RH-4032 (Fig. 2G).

Effects on Microtubule Assembly in Vitro

Experiments with isolated bovine tubulin showed that RH-4032 inhibited the in vitro assembly of tubulin into microtubules in a dose-dependent manner (Fig. 4A). The ability of RH-4032 to inhibit microtubule assembly was characterized by the need for a relatively long pre-incubation period with tubulin prior to initiation of assembly (Fig. 4B).

Covalent Binding to β-Tubulin

Since RH-4032 contains a potentially reactive α-chloroketone moiety, the possibility of covalent binding to a cellular target protein was explored by treating suspension-cultured tobacco cells with [^{14}C]RH-4032, separating the cellular proteins by SDS-PAGE, and detecting radiolabeled protein by autoradiography. This analysis revealed a single labeled protein band that was identified by immunoblotting as β-tubulin, and that migrated slightly slower than α-tubulin (Fig. 5). A similar experiment conducted with isolated bovine tubulin also showed that RH-4032 bound selectively to the β-subunit (Fig. 6), which in this case was the faster-running of the two subunits. Our finding that β-tubulin from tobacco migrated slower than α-tubulin on SDS-PAGE, whereas for bovine tubulin the β-subunit ran faster than the α-subunit, is consistent with data from other workers (Hussey and Gull, 1985; Mizuno et al., 1985).

In light of the unusual ability of RH-4032 to bind covalently and with high specificity to tubulin in tobacco suspension-cultured cells, as well as its high potency, experiments were conducted to explore the possibility of developing a cell-based competitive-binding assay that could detect other antitubulin compounds using [^{3}H]RH-4032 as the radioligand probe. Specific binding of [^{3}H]RH-4032 to the cells, defined as that fraction of the total binding that is inhibited by an excess of unlabeled ligand, was found to increase linearly for approximately 30 min (Fig. 7). In subsequent experiments a 20-min incubation was employed to calculate the rate of binding. Non-specific binding was determined by measuring the amount of [^{3}H]RH-4032 bound during a 20-min incubation following pre-incubation of the cells for 20 min in 100 μM unlabeled RH 4032, and specific binding was calculated by subtracting non-specific binding from total binding. Specific binding saturated in a concentration dependent manner (Fig. 8A) with one-half-maximal binding at 1.1 μM. Scatchard analysis of the specific binding data produced a linear plot, consistent with a single set of binding sites (Fig. 8B).

Effects of Antimicrotubule Agents on Binding of [^{3}H]RH-4032 in Whole Cells

Binding of [^{3}H]RH-4032 was affected in a dose-dependent manner by pre-incubation of the cells with unlabeled RH-4032. The dose-response curve for the effect of unlabeled RH-4032 on binding of the radioligand (Fig. 9A) was characterized by a slight enhancement of binding in the 0.05 to 0.2 μM range, and potent inhibition of binding at higher concentrations (≥0.5 μM). Two other benzamide antimicrotubule agents, pronamide and zarilamide, and the *N*-phenylcarbamate, chlorpropham, also inhibited radioligand binding and in each case slightly lower concentrations than those required for inhibition produced a slight increase in binding (Fig. 9, B–D). The concentration range over which each compound influenced [^{3}H]RH-4032 binding correlated well with the concentration needed to arrest mitosis as measured by an increase in the mitotic index. Pre-incubation of cells with the dinitroaniline, trifluralin, and the phosphoric amide, amiprophosmethyl, at less than 1 μM slightly enhanced binding of [^{3}H]RH-4032, but inhibition of binding was not detected even at a concentration of 100 μM, which for both compounds exceeded by approximately 10-fold the concentration needed to produce a maximal increase in the mitotic index (Fig. 9, F and G). Pre-incubation of cells with paclitaxel resulted in inhibition of [^{3}H]RH-4032 binding (Fig. 9E) at concentrations less than 1 μM. At a range of lower paclitaxel concentrations (0.0016–1 μM), the increase in radioligand binding observed with other antimicrotubule agents was not detected.

The utility of the [^{3}H]RH-4032 tobacco binding assay for quantifying activity of antitubulin agents was explored for a series of benzamide (Table II) and a series of *N*-phenylcarbamate (Table III) analogs by comparing potency of compounds in the binding assay with their ability to inhibit root elongation. An excellent correlation was found between potency in the binding assay and biological activity for the benzamide compounds (Fig. 10). Data for the *N*-phenylcarbamates also suggested a correlation between

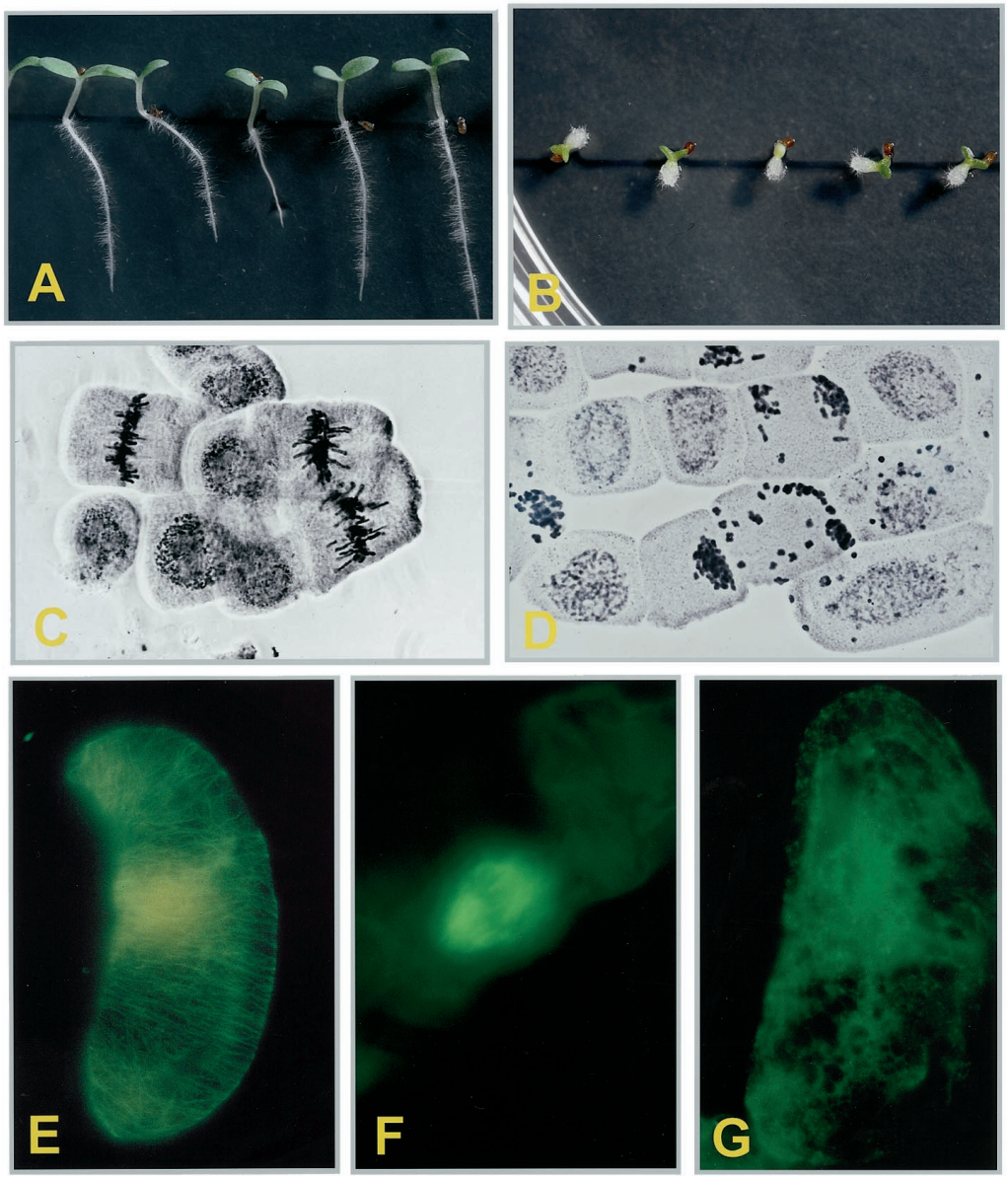


Figure 2. Effects of RH-4032 on root growth, mitosis, and the microtubule cytoskeleton. A, Untreated control seedlings. B, Seedlings germinated in 0.62 μM RH-4032. C, Untreated control root tip cells undergoing mitosis. D, Root tip cells in arrested metaphase following treatment with 0.31 μM RH-4032. Immunofluorescent staining of microtubules in untreated control suspension-cultured cells showing cortical (E) and spindle (F) microtubules. G, Absence of microtubules in cells treated for 3 h with 1 μM RH-4032.

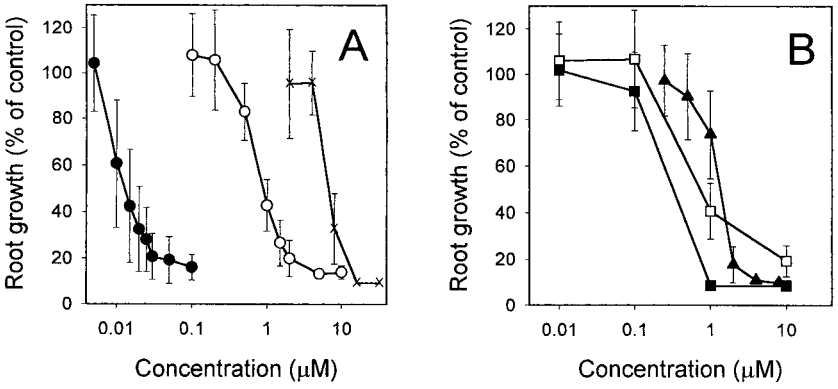


Figure 3. Inhibition of tobacco root elongation by RH-4032 and various antimicrotubule agents. A, Dose-response curves for RH-4032 (●), pronamide (○), and zarilamide (×). B, Dose-response curves for amiprophos-methyl (■), chlorpropham (□), and trifluralin (▲).

Table I. *Effect of RH-4032 (10 μM) on the mitotic index in suspension-cultured tobacco cells as a function of treatment time*

Treatment	Time	Mitotic Index
	h	
Control	0	1.1
RH-4032	1	2.7
RH-4032	3	6.6
RH-4032	6	9.3
RH-4032	9	10.9
Control	9	0.6

potency in the binding assay and ability to inhibit root elongation since the most active analogs in the binding assay (compounds 1–4) displayed the highest biological activity, whereas the inactive analogs in the binding assay (IC50 [concentration of compound required to inhibit binding of the radioligend by 50%] ≥ 200 μM) did not inhibit root elongation.

DISCUSSION

The morphological effects of RH-4032 on plant roots strongly resembled those of herbicides such as trifluralin, amiprophos-methyl, chlorpropham, and pronamide, which inhibit mitosis by disrupting microtubules (Vaughn and Lehnen, 1991). Inhibition of mitosis by RH-4032 was confirmed by the observation of an accumulation of cells in arrested metaphase. The loss of cellular microtubules in treated cells and the ability of RH-4032 to inhibit the in vitro assembly of bovine microtubules showed microtubule disruption to be the cause of mitotic arrest. Although RH-4032 was found to inhibit the assembly of isolated mammalian tubulin, the concentrations needed for inhibition of assembly were much higher than those needed to arrest mitosis in plant cells. This is consistent with the relative sensitivities of plant and mammalian cells to the compound. Cytotoxicity tests against 59 mammalian tumor cell lines at the National Cancer Institute have shown that RH-4032 is about two orders of magnitude less active toward the most sensitive mammalian cells (National Cancer Institute, unpublished results) than it is toward tobacco cells. Although much less active toward mammalian cells than against plant cells, the ability of RH-4032 to inhibit the assembly of brain microtubules in vitro and its activity against mammalian cell lines is of interest since, with the exception of paclitaxel, agents with high potency toward plant microtubules have not been reported to show significant activity in mammalian systems (Morejohn and Foskett, 1986).

RH-4032 was found to bind covalently to the β-subunit of tubulin both in whole cell-labeling studies and in experiments using isolated tubulin. The fact that RH-4032 bound only to β-tubulin and not to other cellular proteins in whole cells indicates a highly specific interaction. This specificity is also reflected in the absence of binding to α-tubulin, despite its high degree of homology to β-tubulin. Although other compounds have been shown to inhibit microtubule assembly by covalent binding to tubulin (Luduena and Roach, 1991), such compounds generally modify multiple Cys residues in tubulin as well as cysteines in other cellular proteins. One exception is the antitumor sulfonamidobenzene compound, T138067, which reacts selectively with Cys-239 of β-tubulin in whole cells (Shan et al., 1999). 2,4-Dichlorobenzyl thiocyanate also reacts preferentially with this Cys residue, but does label multiple proteins when applied to intact cells (Bai et al., 1989). The inhibition of bovine microtubule assembly in vitro by RH-4032 required a relatively long pre-incubation with tubulin, suggestive of a rather slow binding reaction. It is interesting that the need for a long pre-incubation with tubulin has also been described for inhibition of microtubule assembly by 2,4-dichlorobenzyl thiocyanate (Abraham et al., 1986). RH-4032 is the first agent found to bind covalently with high specificity to tubulin in plant cells. The site of covalent binding is currently being explored.

Selective, covalent binding of RH-4032 to tubulin in tobacco suspension-cultured cells enabled the development of a competitive-binding assay in whole cells since it was possible to remove unbound [^{3}H]RH-4032 by extensive washing without loss of the bound ligand. The dose-response curve for the effect of unlabeled RH-4032 on binding of [^{3}H]RH-4032 was characterized by a slight enhancement of binding at

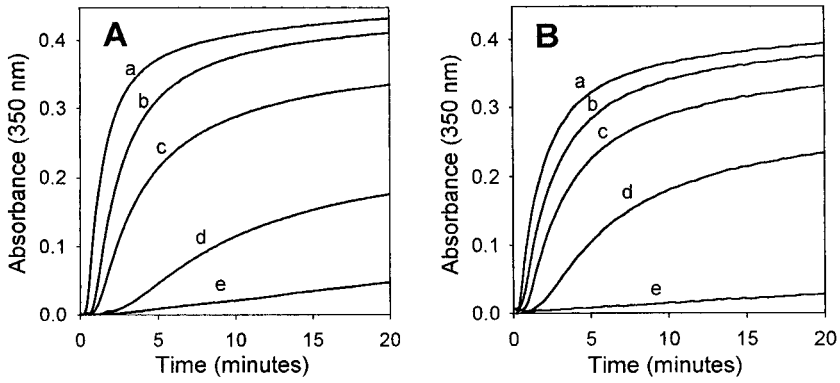


Figure 4. Inhibition of microtubule assembly by RH-4032. A, Isolated bovine tubulin (1 mg/mL) was incubated for 4 h with RH-4032 at 0 μM (a), 3.12 μM (b), 6.25 μM (c), 12.5 μM (d), and 25 μM (e) before initiation of microtubule assembly. B, Tubulin was incubated with dimethylsulfoxide (DMSO) for 1 h (control [a]) or RH-4032 (16 μM) for 1 h (b), 2 h (c), 4 h (d), and 6 h (e) before induction of microtubule assembly. The extent of assembly in the control treatment was unaffected by the length of pre-incubation over the 1- to 6-h time period.

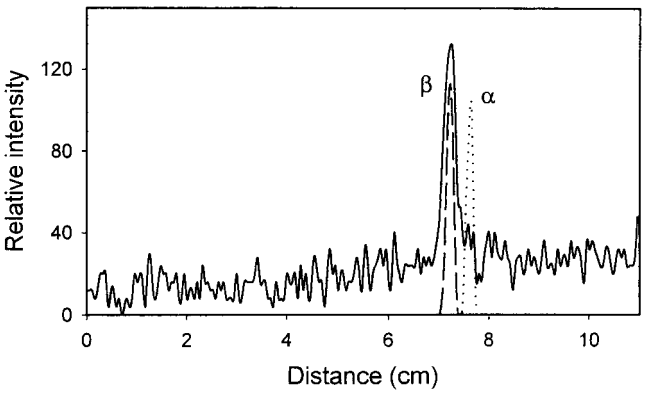


Figure 5. Analysis of radiolabeled protein by SDS-PAGE and autoradiography following treatment of tobacco suspension-cultured cells with [^{14}C]RH-4032. The separated proteins were analyzed by autoradiography (solid line) and by immunoblotting using antibodies to α- (dotted line) and β-tubulin (dashed line).

concentrations that produced a submaximal increase in the mitotic index and strong inhibition of binding at higher concentrations. The inhibition of binding at higher concentrations presumably reflects competitive inhibition of radioligand binding. The enhancement of radioligand binding observed at lower RH-4032 concentrations was also found for the other agents that cause disassembly of microtubules (pronamide, zarilamide, trifluralin, amiprophos-methyl, and chlorpropham). That for each compound the enhanced binding occurred at concentrations comparable with those needed to inhibit mitosis suggests that it is a consequence of cellular microtubule disruption. A possible explanation for the ability of lower concentrations of RH-4032 and the other agents to enhance radioligand binding is that subs-

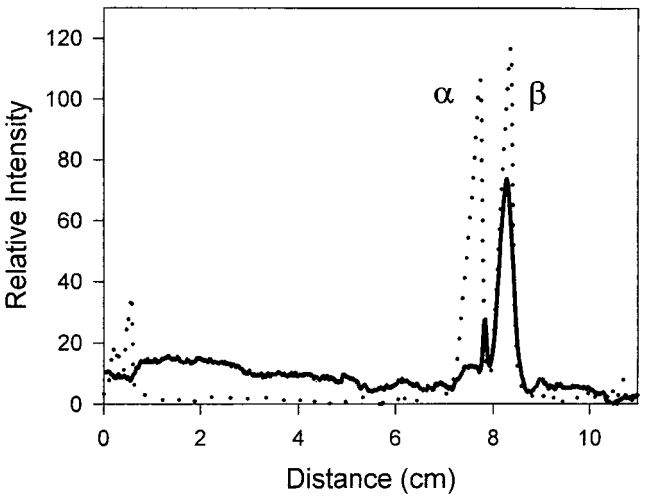


Figure 6. Analysis of radiolabeled protein by SDS-PAGE and autoradiography following treatment of isolated bovine tubulin with [^{14}C]RH-4032. After SDS-PAGE, the separated proteins were analyzed by autoradiography (solid line) and by immunoblotting using antibodies to α- and β-tubulin. A duplicate gel was stained for protein (dotted line).

toichiometric amounts of ligand may be sufficient to promote disassembly of cellular microtubules and produce a higher concentration of disassembled tubulin in the cell that is available for radioligand binding. Such an effect might be expected if RH-4032 and the other agents act by a "tip-poisoning" mechanism similar to that proposed for colchicine (Margolis and Wilson, 1977); it is believed that colchicine binds to disassembled tubulin and inhibits microtubule assembly at substoichiometric concentrations by the ability of the colchicine-tubulin complex to bind to the growing end of the microtubule and prevent elongation. Although the hypothesis that RH-4032 and the other agents cause microtubule disassembly by this mechanism is speculative, the fact that the microtubule-stabilizing drug paclitaxel, which also inhibited binding of [^{3}H]RH-4032, did not enhance binding at lower concentrations is consistent with this explanation.

It is known that paclitaxel binds to the β-tubulin subunit (Rao et al., 1995; Nogales et al., 1998). The inhibition of [^{3}H]RH-4032 binding by paclitaxel could reflect a common binding site, or an allosteric effect on binding of the radioligand. However, an alternative and perhaps more plausible explanation, which is consistent with the hypothesis that RH-4032 binds to the disassembled form of tubulin, is that paclitaxel reduces the cellular concentration of disassembled tubulin available for binding of the radioligand by its ability to promote microtubule assembly.

The ability of the other benzamide antimicrotubule agents, pronamide and zarilamide, to inhibit binding of [^{3}H]RH-4032 and their structural similarity (Fig. 1) suggest a common binding site on β-tubulin. The precise mechanism by which *N*-phenylcarbamate herbicides disrupt microtubule function has been the subject of debate. Although it has been proposed that these compounds act on microtubule organizing cen-

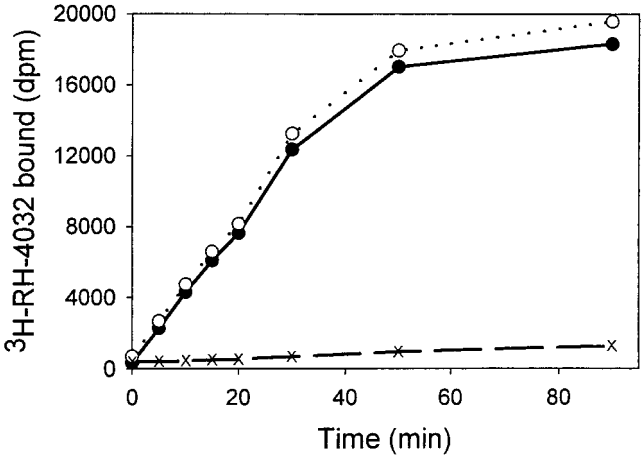


Figure 7. Time course for binding of [^{3}H]RH-4032 in tobacco suspension-cultured cells. Cells were treated with [^{3}H]RH-4032 (0.25 μM, 604 mCi/mmol) for different times, and analyzed for bound radioactivity as described in "Materials and Methods." Total binding, ○; non-specific binding, X; and specific binding, ●.

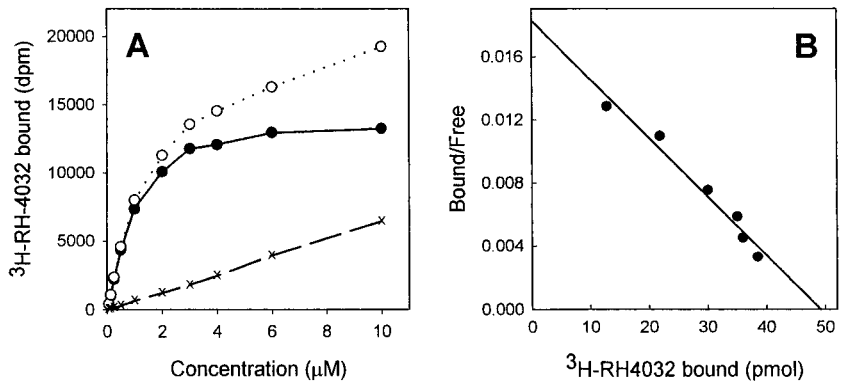


Figure 8. Saturable binding of [^{3}H]RH-4032 in tobacco suspension-cultured cells. Cells were treated with [^{3}H]RH-4032 (151 mCi/mmol) at various concentrations for 20 min, and analyzed for bound radioactivity. A, Total binding, ○; nonspecific binding, X; and specific binding, ●. B, Scatchard analysis of the specific binding data shown in A.

ters rather than by binding to tubulin (Coss et al., 1975), the use of immobilized ethyl *N*-phenylcarbamate to purify plant tubulin suggests that the compounds do in fact bind tubulin (Mizuno et al., 1981). In the present study *N*-phenylcarbamates inhibited binding of [^{3}H]RH-4032, and the ability of different analogs to inhibit binding appeared to correlate with their biological activity. The most likely explanation for these results is that *N*-phenylcarbamates bind to tubulin at the benzamide site.

Since the dinitroaniline herbicide, trifluralin, and the phosphoric amide herbicide, amiprophos-methyl, did not inhibit binding of [^{3}H]RH-4032, even at concentrations considerably in excess of those needed to increase the mitotic index, these agents must bind to a different site from RH-4032. It is believed that trifluralin and amiprophosmethyl bind to α-tubulin and do not share the same binding site as pronamide since tubulin mutations that confer resistance to these herbicides were found to occur on the α-subunit and did not affect sensitivity to pronamide (Anthony and Hussey, 1999). Such a model is consistent with the observed lack of inhibition of [^{3}H]RH-4032 binding by trifluralin and amiprophosmethyl in the present study.

The present results show that RH-4032 can be used as a tubulin-binding probe in a cell-based competitive-binding assay to study effects of antimicrotubule agents. This offers a new approach to studying cellular microtubules and the effects of antimicrotubule agents. The vast majority of studies of antimicrotubule agents have employed mammalian brain tubulin due to the difficulty in isolating sufficient tubulin from other sources. Mammalian brain tubulin is not a satisfactory model for tubulin from other sources since the ability of many drugs and agrochemicals to interact with tubulin is known to be highly dependent on the source of tubulin (Morejohn and Foskett, 1986). Although tubulin has been isolated from plants (Mizuno et al., 1981, 1985; Morejohn and Foskett, 1986) the amounts obtained have not been sufficient for studies such as the screening of compound libraries for antimicrotubule effects. Thus a major advantage of the [^{3}H]RH-4032 cell-based binding assay is that antimicrotubule effects can be studied in a plant cell without the need for isolated tubulin. The assay also provides a system that accounts for effects of cellular uptake and metabolism of a compound on its antimicrotubule activity.

In addition to plants, benzamides of the RH-4032 class are highly active toward other organisms, including fungi, algae, protozoan, and mammalian cells, although structure-activity relationships within this series of compounds differ somewhat depending on the organism. A similar binding assay to the tobacco assay described here has been developed in the Oomycete fungus, *Phytophthora capsici*, using the analog RH-7281 as the radioligand (D.H. Young and R.A. Slawecki, manuscript in preparation), and studies are in progress to develop cell-based binding assays for other cell types using appropriate analogs.

MATERIALS AND METHODS

Chemicals

RH-4032 [3, 5-dichloro-*N*-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-benzamide], zarilamide (4-chloro-*N*-[cyano-(ethoxy)-methyl]benzamide), amiprophosmethyl, and the *N*-phenylcarbamate compounds, A through G were synthesized at Rohm and Haas Company. Trifluralin, chlorpropham, chlorbufam, barban, and pron-amide were obtained from Riedel de Haen (Hannover, Germany). Paclitaxel was obtained from Calbiochem (San Diego).

Inhibition of Tobacco (*Nicotiana tabacum*) Root Elongation

Aliquots (20 μL) of test compounds dissolved in DMSO were added to 20 mL of nutrient medium consisting of Murashige and Skoog salts (Murashige and Skoog, 1962), 2% (w/v) Suc, and 1% (w/v) agar at 50°C. The molten agar was poured immediately into 9-cm diameter Petri plates. After the agar had solidified, surface-disinfested tobacco cv Samsun seeds, were planted on the agar (20 seeds per treatment). Plates were incubated in a vertical position at 27°C for 7 d with a 12-h photoperiod, then the lengths of the seedling roots were measured and EC50 values (concentration of compound required to inhibit root elongation by 50%) were determined from dose-response curves.

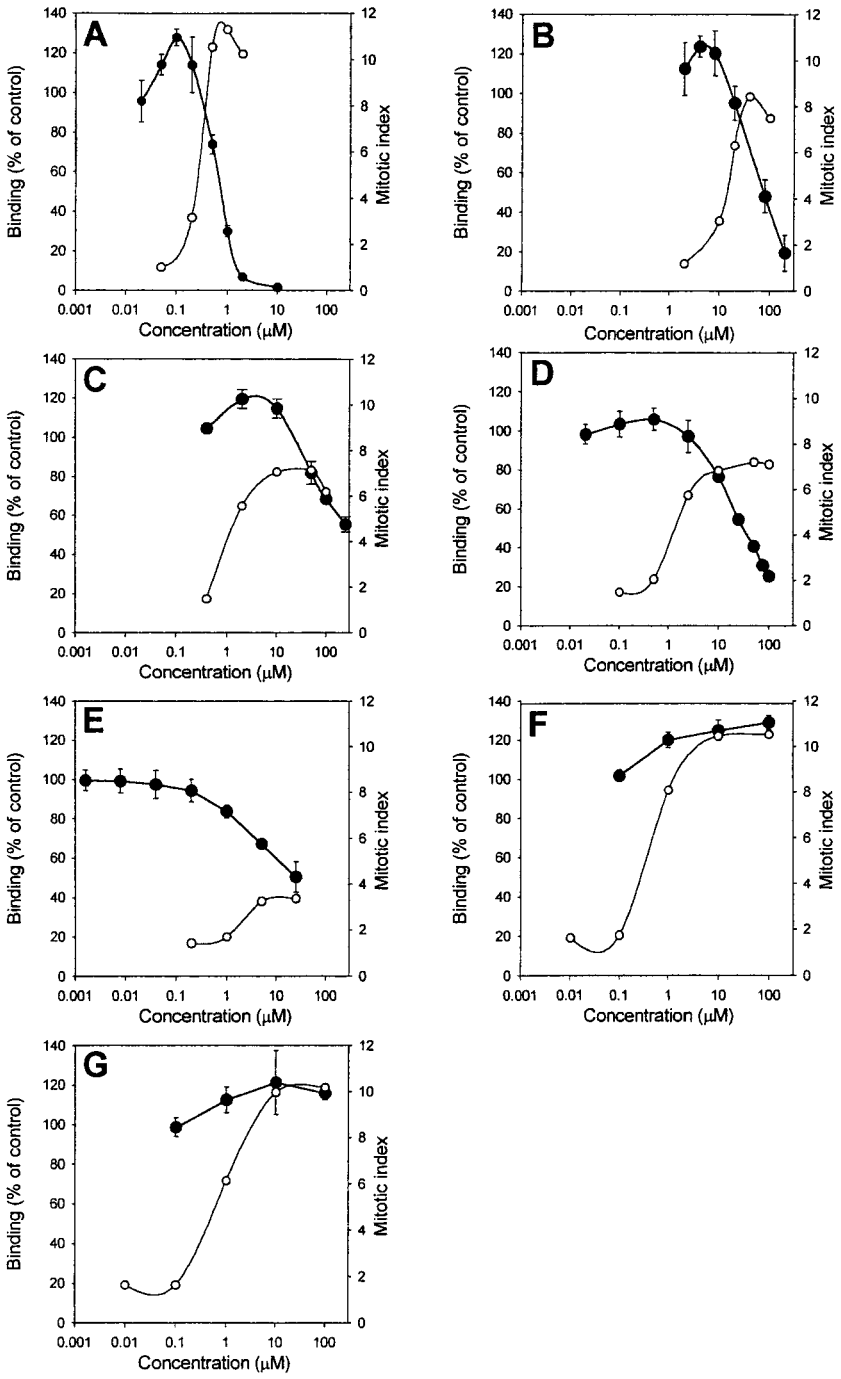
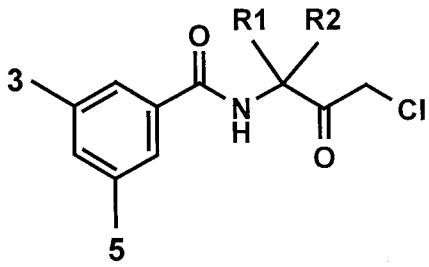


Figure 9. Effects of antimicrotubule agents on binding of [^{3}H]RH-4032 (●) and the mitotic index (○) in tobacco suspension-cultured cells. Values for binding are the means of three replicates, and error bars represent SD. A, RH-4032; B, zarilamide; C, pronamide; D, chlorpropham; E, paclitaxel; F, trifluralin; and G, amiprophosmethyl.

Chromosome Staining

Effects on mitosis in tobacco root tips were studied by treating young hydroponically grown tobacco plants with RH-4032 for 18 h. Root tip squashes were prepared by fixation in Carnoy's fluid (6:3:1 of 95% [w/v] ethanol: chloroform:glacial acetic acid) followed by hydrolysis in 1 N HCl at 60°C for 15 min. The cells were then stained in Feulgen stain for 1 h and mounted in 1% (w/v) acetocarmine. Effects on mitosis in suspension-cultured tobacco cells were studied using cells cv Xanthi, grown at 27°C on a gyrotary shaker at 200 rpm in a modified Linsmaier and Skoog medium (Nagata et al., 1981). Cells were pelleted by centrifugation at 1,000*g*, resuspended in fresh medium using 19 mL of medium per milliliter of pelleted cells, and allowed to grow for 40 h to ensure a population of rapidly dividing cells. Cells were recentrifuged, resuspended in fresh medium at 9 mL of medium per milliliter of pelleted cells, and dispensed as 2-mL aliquots in 25-mL glass vials. Test compounds were added to the vials as 5-μL aliquots of solutions in DMSO. Untreated controls received 5 μL of

Table II. *Inhibition of [^{3}H]RH-4032 binding in tobacco suspension-cultured cells and inhibition of tobacco root elongation by various benzamides*



Compound	3	5	R1	R2	Root Inhibition EC50	Binding Assay IC50
					μM	
1	Cl	Cl	CH_3	C_2H_5	0.014	0.73
2	Br	CH_3	CH_3	C_2H_5	0.026	2.7
3	Cl	Cl	CH_3	CH_3	0.019	4.5
4	Cl	CH_3	CH_3	C_2H_5	0.037	7.4
5	F	CF_3	CH_3	C_2H_5	0.055	14.3
6	Cl	H	CH_3	CH_3	0.088	33.7
7	Cl	H	CH_3	C_2H_5	0.089	64.0
8	Cl	CH_2OCH_3	CH_3	C_2H_5	0.16	99.6
9	Cl	Cl	C_2H_5	C_2H_5	0.453	141.6

DMSO alone. Vials were incubated with shaking at 27°C for 9 h, then fixed in formic acid (Bayliss and Gould, 1974). After mounting in 1% (w/v) acetocarmine, the mitotic index was determined as the percentage of cells in metaphase based on examination of 2,000 cells per treatment.

Immunofluorescence Microscopy of Microtubules in Tobacco

Suspension-cultured cells were treated with RH-4032 as above before processing for immunofluorescence microscopy as described previously (Young, 1991) using the primary antibody YL 1/2 (Kilmartin et al., 1982) and a goat anti-rat IgG fluorescein isothiocyanate conjugate as the secondary antibody.

Microtubule Assembly Assay

Tubulin was isolated from bovine brain by two cycles of assembly/disassembly (Tiwari and Suprenant, 1993). Assay mixtures contained 1.0 mg/mL of tubulin in 1 M Na Glu, pH 6.6, 1 mM $MgCl_2$, and either RH-4032, added as a solution in DMSO, or DMSO alone (control). The final DMSO concentration in the assay was 2% (v/v). Assay mixtures were incubated at 37°C for the appropriate time, then chilled on ice for 5 min. Polymerization of tubulin was initiated by addition of GTP (0.1 mM), and incubation of the sample at 37°C. Assembly was followed by monitoring the increase in A_{350} for 20 min using a temperature-controlled cell in a UV-2401PC spectrophotometer (Shimadzu, Columbia, MD).

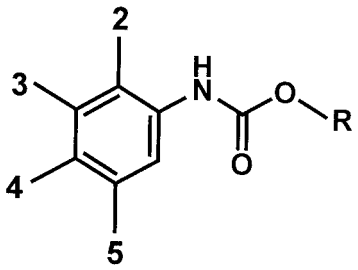
Labeling of Protein in Tobacco Cells with [^{14}C]RH-4032

Suspension-cultured cells were treated with 10 μM [^{14}C]RH-4032 (specific activity 21.4 mCi/g) for 1 h, then collected by filtration. The cells were frozen in liquid nitrogen and ground to a fine powder. Proteins were extracted and prepared for SDS-PAGE by heating for 5 min at 100°C in SDS-PAGE sample buffer (62 mM Tris [tris-(hydroxymethyl)aminomethane]-HCl buffer, pH 6.8, containing 3% [w/v] SDS [L-5750, Sigma, St. Louis], 5% [w/v] mercaptoethanol, and 10% [w/v] glycerol). The extracted proteins were subjected to SDS-PAGE (Laemmli, 1970) on a 7.5% (w/v) polyacrylamide gel, then the separated proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was sprayed with EN^{3}HANCE spray (DuPont NEN Research Products, Boston) and the location of radiolabeled protein was determined by autoradiography using film (X-Omat AR, Kodak, Rochester, NY) with a 3-week exposure. The α- and β-tubulin subunits were detected using the monoclonal antibodies, YL 1/2 and N357 (Amersham, Buckinghamshire, UK), respectively, and a gold-labeled secondary antibody.

Labeling of Isolated Bovine Tubulin with [^{14}C]RH-54032

Tubulin (10 μM) in 1.0 M sodium Glu, pH 6.6, containing 1 mM $MgCl_2$ was incubated with 25 μM [^{14}C]RH-4032 for 4 h at 37°C. After cooling on ice, tubulin was precipitated by an addition of 20% (w/v) trichloroacetic acid and incubated on ice for a further 20 min. After centrifugation at 12,000*g* for 5 min the tubulin pellet was washed once with 80% (w/v) ice-cold acetone, dissolved in SDS-PAGE sample buffer at room temperature, heated for 2 min at 100°C, then subjected to SDS-PAGE on duplicate 7.5% (w/v) polyacrylamide gels. One gel was stained for protein with Coomassie Brilliant Blue R250, and proteins from the second gel were transferred to nitrocellulose and analyzed by

Table III. *Inhibition of [^{3}H]RH-4032 binding in tobacco suspension-cultured cells and inhibition of tobacco root elongation by various N-phenylcarbamates*



Compound	2	3	4	5	R	Root Inhibition EC50	Binding Assay IC50
						μM	
Chlorbufam	H	Cl	H	H	-CH(CH_3)CCH	1.18	18.6
Chlorpropham	H	Cl	H	H	-isopropyl	0.75	29.8
A	H	Cl	H	H	-$CH_2CCCH_2CH_3$	0.99	39.2
Barban	H	Cl	H	H	-CH_2CCCH_2Cl	1.04	47.9
B	H	Cl	H	Cl	-isopropyl	14.4	81.8
C	H	Cl	Cl	H	-isopropyl	117.1	116
D	H	Cl	H	Cl	-CH_3	34.5	199
E	Cl	Cl	H	H	-isopropyl	>200	>200
F	Cl	H	Cl	Cl	-isopropyl	>200	>200
G	H	Cl	CH3	H	-ispropyl	>200	>200

autoradiography and immunoblotting as described above. The α- and β-tubulin subunits were detected using the monoclonal antibodies, N356 and N357 (Amersham), respectively.

Assay for Binding of [^{3}H]RH-4032 in Tobacco Cells

Suspension-cultured cells from a 6-d-old culture were pelleted by centrifugation at 1,000*g*, and resuspended in fresh medium using 9 mL of medium/mL of pelleted cells. Aliquots (2 mL) of cell suspension were added to 25-mL-capacity glass vials. [^{3}H]RH-4032 was added as 5 μL of solutions in DMSO to give the desired concentrations. Vials were incubated with shaking at 27°C for 20 min, then binding of the radioligand was stopped by adding 5 μL of unlabeled 40 mM RH-4032. The samples were incubated with shaking at 27°C for a further 20 min, then transferred to 15-mL-capacity polypropylene centrifuge tubes in an ice bath. Each vial was rinsed with 2 mL of ice-cold medium, which was pooled with the rest of the sample. Following centrifugation at 4°C for 3 min at 2,500 rpm the supernatant was discarded and the pelleted cells were resuspended in 4 mL of ice-cold 10% (w/v) trichloroacetic acid. Samples were kept for 1 h on ice, centrifuged again, and the cells resuspended in 4 mL of ice-cold ethanol. After incubation for an additional 1 h on ice, the cells were collected by filtration on glass fiber filters using a multi-probe cell harvester (Brandel, Gaithersburg, MD) and washed twice with 10 mL of ice-cold ethanol. The filters with washed cells were transferred to scintillation vials, 10 mL of Hydrofluor scintillation fluid was added to each, and the samples were counted in a scintillation counter to determine the amount of bound radioactivity. Non-specific binding was determined by pre-incubation of the cells for 20 min with 100 μM unlabeled RH-4032 prior to addition of the radioligand.

In experiments designed to test the effect of antimicrotubule agents on binding of the radioligand, vials containing 2 mL of cells received 5 μL of DMSO (control) or 5 μL of the antimicrotubule agent dissolved in DMSO. The vials were then incubated with shaking at 27°C for 2 h prior to the addition of [^{3}H]RH-4032 (0.25 μM, 604 mCi/mmol) and processed as described above.

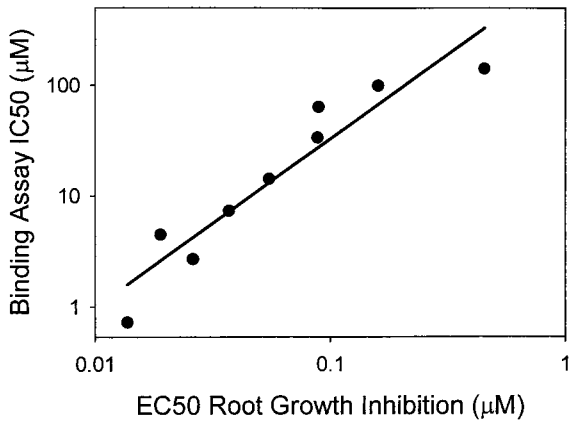


Figure 10. Correlation between ability to inhibit binding of [^{3}H]RH-4032 in tobacco suspension-cultured cells and ability to inhibit root elongation in tobacco seedlings for a series of benzamide analogs. Logarithmic plot of IC50 values for inhibition of binding versus EC50 values for inhibition of root elongation for the compounds listed in Table II. The R^2 value for the correlation was 0.89.

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