Amiprophos-methyl (APM), a phosphoric amide herbicide, was previously reported to inhibit the in vitro polymerization of isolated plant tubulin (L.C. Morejohn, D.E. Fosket [1984] Science 224: 874-876), yet little other biochemical information exists concerning this compound. To characterize further the mechanism of action of APM, its interactions with tubulin and microtubules purified from cultured cells of tobacco (Nicotiana tabacum cv Bright Yellow-2) were investigated. Low micromolar concentrations of APM depolymerized preformed, taxol-stabilized tobacco microtubules. Remarkably, at the lowest APM concentration examined, many short microtubules were redistributed into fewer but 2.7-fold longer microtubules without a substantial decrease in total polymer mass, a result consistent with an end-to-end annealing of microtubules with enhanced kinetic properties. Quasi-equilibrium binding measurements showed that tobacco tubulin binds [3H]oryzalin with high affinity to produce a tubulin-oryzalin complex having a dissociation constant ($K_d$) = 117 nM (pH 6.9; 23°C). Also, an estimated maximum molar binding stoichiometry of 0.32 indicates pharmacological heterogeneity of tobacco dimers and may be related to structural heterogeneity of tobacco tubulin subunits. APM inhibits competitively the binding of [3H]oryzalin to tubulin with an inhibition constant ($K_i$) = 5 μM, indicating the formation of a moderate affinity tubulin-APM complex that may interact with the ends of microtubules. APM concentrations inhibiting tobacco cell growth were within the threshold range of APM concentrations that depolymerized cellular microtubules, indicating that growth inhibition is caused by microtubule depolymerization. APM had no apparent effect on microtubules in mouse 3T3 fibroblasts. Because cellular microtubules were depolymerized at APM and oryzalin concentrations below their respective $K_d$ and $K_i$ values, both herbicides are proposed to depolymerize microtubules by a substoichiometric endwise mechanism.

Phosphoric amide herbicides such as APM (Tokunol M) and butamiphos (Cremart) were developed as preemergence herbicides and are effective on annual grasses and broadleaf weeds (Aya et al., 1975). The uses of phosphoric amides are similar to those of the dinitroaniline herbicides, including oryzalin (Surflan) and trifluralin (Treflan) (Ashton and Crafts, 1981). The structures of oryzalin and APM are given in Figure 1. Both APM and oryzalin produce gross morphological effects in plants, the most characteristic of which is swollen root tips (Sumida and Ueda, 1976; Upadhyaya and Nooden, 1977; Ashton and Crafts, 1981). These herbicides halt plant growth by inhibiting cell division and elongation; the orientation of cellulose microfibril deposition in the cell wall becomes random, producing cells having more isodiametric shapes typical of “tumor” roots. These cellular activities are disrupted because of the depolymerization of microtubules, which usually direct the polar distribution of subcellular components (Morejohn, 1991). These and other observations have provided ample evidence that microtubules are essential subcellular determinants of morphogenesis in plants (Lloyd, 1991).

Microtubules are filamentous polymers consisting mainly of tubulin, a protein heterodimer with similar $\alpha$ and $\beta$ subunits, each having a molecular mass of about 50 kD. Dimeric tubulin polymerizes in a head-to-tail manner to form 13 linear protofilaments within the microtubule wall, and a dynamic equilibrium is maintained between microtubules and the soluble pool of tubulin dimers. Kinetic properties of microtubules, such as dynamic instability and treadmilling, have been observed mainly in animal microtubules (Gelfand and Bershadsky, 1991). Plant cells have several functionally distinct microtubule arrays that form at particular stages of the cell cycle (Lloyd, 1991), and plant microtubules have unique in vitro polymerization characteristics (Bokros et al., 1993; Hugdahl et al., 1993).

Studies of the mechanisms of action of antimicrotubule herbicides offer crucial information concerning the structure and function of plant tubulin and the regulation of plant microtubule dynamics and thus are important to our understanding of plant cell growth and differentiation. In contrast to the vast data available regarding drugs that bind to mammalian tubulin (Wilson and Jordan, 1993), very little information exists concerning the pharmacology of plant tubulin (Morejohn, 1991). Our studies have demonstrated that plant tubulin binds both colchicine and oryzalin, although their __________

Abbreviations: APM, amiprophos-methyl; DAPI, 4',6-diamidino-2-phenylindole; IB, isolation buffer; $K_{dep}$, microtubule depolymerization constant; NP-40, Nonidet P-40; $r$, molar binding ratio (mol oryzalin bound per mol tubulin); $t$, threshold concentration; TA, tubulin-APM; TC, tubulin-colchicine; TO, tubulin-oryzalin.
Binding characteristics are very different (Morejohn et al., 1987a; Hugdahl and Morejohn, 1993). Colchicine binds slowly and with very low affinity ($K_d = 1.03 \text{ mM}$) to plant tubulin to form a TC complex that does not readily dissociate into unliganded, tubulin and free colchicine, thus exhibiting "tight" binding characteristics (Morejohn et al., 1984, 1987b). In contrast, oryzalin binds rapidly and with high affinity ($K_d = 97 \text{ nM}$) to plant tubulin to form a TO complex that readily dissociates into unliganded tubulin and free oryzalin (Hugdahl and Morejohn, 1993). TC and TO are proposed to inhibit tubulin assembly by co-polymerizing with unliganded tubulin and inhibiting further tubulin polymerization (Strachen and Hess, 1983; Morejohn and Fosket, 1984a; Morejohn et al., 1987a, 1987b; Hugdahl and Morejohn, 1993). However, the number of different drug-binding sites on plant tubulin is not known, and it has not been determined whether different classes of antimicrotubule herbicides bind to the same or different sites on plant tubulin (Morejohn, 1991).

Although a number of studies have used phosphoric amides as probes of microtubule function in cells of algae (Kiermayer and Fedtke, 1977; Quader and Filner, 1980; La Claire, 1987; Mizuta et al., 1989; Schibler and Huang, 1991; James et al., 1993), protostysts (Pape et al., 1991), and advanced land plants (Mita and Shibaoka, 1983; Bajer and Mole'-Bajer, 1986; Falconer et al., 1988), only one report exists concerning the in vitro interaction of phosphoric amides with plant tubulin (Morejohn and Fosket, 1984b). APM at low micromolar concentrations was shown to inhibit the taxol-induced polymerization of tubulin isolated from cultured cells of rose (Rosa sp.), with no effect on mammalian tubulin assembly. Turbidimetric measurements demonstrated that APM inhibits both the rate and extent of microtubule formation, and EM revealed fewer and shorter microtubules with increased APM concentrations. Polymer sedimentation assays confirmed that APM causes a concentration-dependent decrease in rose tubulin polymer mass (Morejohn and Fosket, 1984b). While these results showed an interaction of APM with plant tubulin, the experiments were conducted using a buffer containing 1 mM Suc, which itself was found subsequently to inhibit both the rate and extent of taxol-induced plant tubulin polymerization (Bokros et al., 1993).

Furthermore, Suc stabilizes preformed microtubules, making an examination of the effects of APM on preformed microtubules unfeasible. Finally, because Suc increases solution viscosity substantially and tubulin polymerization is altered at least in part by slowed dimer and polymer diffusion rates, it was important to reexamine the interaction of APM with plant tubulin in an assembly buffer not containing Suc.

The primary purpose of this study was to characterize in greater detail the interaction of APM with purified plant tubulin and microtubules in solutions not containing Suc and, when appropriate, to compare the results to those obtained in parallel experiments with oryzalin. We used tobacco BY-2 cells (Nicotiana tabacum cv Bright Yellow-2), widely adopted as a model plant cell line (Nagata et al., 1992), as a source of tubulin and for experiments on cellular microtubules.

Materials and Methods

Materials

Analytical grade APM [N-isopropyl O-methyl O-(2-nitro-p-toly)] phosphoramidothioate; Tokunol M] was kindly provided by Dr. Carl Fedtke (Bayer AG, Leverkusen, Germany). Aphidicolin and monoclonal antibody DM1B were from Amersham. Fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody was from Pierce. Sorbitol, BSA, NP-40, DAPI, and N-propyl gallate were from Sigma. Mowiol was obtained from Calbiochem. All other materials were obtained from sources as described previously (Bokros et al., 1993; Hugdahl and Morejohn, 1993).

Suspension Culture of Tobacco Cells

Suspension cultures of tobacco (Nicotiana tabacum, cv Bright Yellow-2) were grown as previously described (Nagata et al., 1992; Bokros et al., 1993), except that 2 mL of cells were transferred to 100 mL of medium at 7-d intervals.

Protein Electrophoresis

One-dimensional SDS-PAGE of pure tobacco tubulin was done according to the method of Studier (1973), and gels were stained with silver.

Ligand-Binding Methods

Quasi-Equilibrium Dialysis Measurements

The binding of [14C]oryzalin to tobacco tubulin was performed with equilibrium dialysis, which provides quasi-equilibrium measurements as described previously (Hugdahl and Morejohn, 1993). All ligand-binding experiments were performed in a tubulin IB consisting of 50 mM Pipes-KOH (pH 6.9), 1 mM EGTA, 0.5 mM MgSO4, and 1 mM DTT, supplemented with 0.1 mM GTP, 2% (v/v) DMSO, 50 μg/mL Na-p-tosyl-l-arginine methyl ester, and 5 μg/mL each of pepstatin A, leupeptin hemisulfate, and aproginin. Details of the technical limitations of [14C]oryzalin binding, including its low aqueous solubility and low specific radioactivity, have been published (Hugdahl and Morejohn, 1993).

Most known and suspected antimicrotubule herbicides have relatively low aqueous solubilities and may bind to the...
Amiprophos-Methyl Binding to Tobacco Tubulin

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surfaces of containers and be lost from solution (Fedtke, 1982; Hugdahl and Morejohn, 1993). Pilot experiments were carried out to determine the solubility characteristics of APM in IB solutions containing 2% (v/v) DMSO. Because a radiolabeled form of APM was not available, the solubility of APM was monitored spectrophotometrically. Maximum absorption of APM in IB occurs at 235 nm (ε = 1.2 x 10⁴), and a linear relationship between A and APM concentration was found at ≤200 μM. The lower limit of detection of APM with A was 50 μM. Various concentrations of APM were incubated for 3 h at 23°C in the dialysis chamber to be used for ligand binding, and APM concentrations were measured at A₂₅₂. It was determined that at APM concentrations >110 μM considerable APM bound to the dialysis chamber and was lost from solution. Thus, all ligand-binding and depolymerization experiments were conducted with APM concentrations well below 110 μM.

Gel-Filtration Chromatography

To test the reversibility of APM binding to tobacco tubulin, two 0.5-mL tubulin samples were preincubated at 23°C for 1 h in the presence or absence of APM and loaded onto separate, identically prepared gel-filtration columns of Sephadex G-10 (bed volume = 1.5 mL) equilibrated with IB containing the supplements indicated above. Samples were chromatographed at a flow rate of 0.8 mL/min at 23°C. After the void volume was discarded, fractions of the eluted protein were collected and assayed for protein, and the first four fractions containing tubulin were retained and recombined to give 3 μM tubulin in each sample.

Indirect Immunofluorescence Microscopy

Microtubules in tobacco cells were stained according to the method of Simmonds et al. (1985), with several modifications. Cells were fixed for 1 h at room temperature in 4% (w/v) paraformaldehyde in IB containing 0.4 M sorbitol, 5 mM EGTA, and the protease inhibitors listed above. Cells were washed three times for 5 min, and cell walls were permeabilized by a 1-h digestion at room temperature in 2% (w/v) cellulysin and 0.2% (w/v) pectolyase in the same buffer. Cells were washed three times in a buffer consisting of 10 mM Tris-HCl, pH 7.4, 5 mM EGTA, and 0.15 M NaCl (TEN) supplemented with protease inhibitors as described above. Cells were settled onto poly-L-Lys-coated slides and extracted in TEN containing 1% (v/v) NP-40. Cells were blocked for 15 min with 2% (w/v) BSA in 0.05% (v/v) NP-40 in TEN and incubated in a humidified chamber for 1 h at room temperature with a 1:500 (v/v) dilution of mouse monoclonal antibody against β-tubulin (DM1B) (Blose et al., 1984) (Amersham) in the same buffer. Cells were washed three times for 5 min with TEN and incubated with a 1:100 (v/v) dilution of fluorescein isothiocyanate-conjugated, rabbit anti-mouse antibody (Pierce). Cells were washed and DNA was stained simultaneously with three rinses in TEN containing 0.2 μg/mL DAPI (Sigma). Cells were mounted under coverslips with Mowiol (Calbiochem) medium containing 2% (w/v) 4N-propyl gallate (Sigma) to prevent fading (Osborn and Weber, 1982). Observations and photographs were made on an Olympus BH-2 fluorescence microscope.

For staining of microtubules in mouse 3T3 fibroblasts, cells were grown and processed as described previously (Hugdahl and Morejohn, 1993). Fibroblast cultures were obtained from Dr. Ben A. Murray (Department of Developmental and Cell Biology, University of California, Irvine).

RESULTS

Depolymerization of Taxol-Stabilized Tobacco Microtubules by APM

Tubulin was isolated from stationary phase cells of tobacco (N. tabacum cv Bright Yellow-2) by DEAE-Sephadex A50 chromatography (Morejohn and Fosket, 1982; Bokros et al., 1993). Microtubules were polymerized by gradual temperature ramping from 0 to 25°C in the presence of taxol and were purified by sedimentation through a Suc cushion (Bokros et al., 1993). The inset in Figure 2 shows silver-stained SDS-PAGE analysis (Studier, 1973) of microtubules and reveals electrophoretic purity of tobacco α- and β-tubulin polypeptides.

The effect of APM on the mass of taxol-stabilized tobacco microtubules was quantified by polymer sedimentation as-

![Figure 2. APM- and oryzalin-induced depolymerization of pure taxol-stabilized tobacco microtubules. The amount of tubulin polymer obtained at each concentration of APM (solid circle) and oryzalin (open circle) is expressed as a percentage of the control microtubule sample having no herbicide. The dashed lines and arrows designate half-maximal depolymerization of taxol-stabilized tobacco microtubules by 15 μM APM (filled arrowhead) and 5.6 μM oryzalin (open arrowhead). The inset shows a silver-stained, 9% polyacrylamide SDS gel (Studier, 1973) containing 10 μg of purified tobacco tubulin (lane 1) and molecular mass markers (lane 2) of 45, 66, 97, and 116 kD.](https://www.plantphysiol.org/)

Figure 2. APM- and oryzalin-induced depolymerization of pure taxol-stabilized tobacco microtubules. The amount of tubulin polymer obtained at each concentration of APM (solid circle) and oryzalin (open circle) is expressed as a percentage of the control microtubule sample having no herbicide. The dashed lines and arrows designate half-maximal depolymerization of taxol-stabilized tobacco microtubules by 15 μM APM (filled arrowhead) and 5.6 μM oryzalin (open arrowhead). The inset shows a silver-stained, 9% polyacrylamide SDS gel (Studier, 1973) containing 10 μg of purified tobacco tubulin (lane 1) and molecular mass markers (lane 2) of 45, 66, 97, and 116 kD.

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Ported previously for taxol-induced tobacco tubulin polymers were assembled, as revealed by EM. Figure 3A shows that in the control sample numerous short microtubules with a mean length of 1.4 ± 0.7 μm and some small polymorphic polymers were assembled, as reported previously for taxol-induced tobacco tubulin polymerization (Bokros et al., 1993). At the lowest concentration of APM tested (4 μM), small polymorphic polymers were absent, and fewer but ≥2.7-fold longer microtubules (3.8 ± 1.6 μm) were observed. This result indicated that a redistribution of microtubule number and length occurred at an APM concentration at which ≥90% of polymer was estimated to be assembled (Fig. 2). Figure 3B shows long microtubules with a mean length of 4.8 ± 3.2 μm after treatment with 6 μM APM, and Figure 3C also shows long microtubules (4.6 ± 1.9 μm) present after treatment with 15 μM APM. The summary of microtubule length measurements at different APM concentrations in Table 1 shows that all APM-treated samples had microtubules significantly longer than the control.

Another notable structural feature of APM-treated microtubules is the intermittent lattice discontinuities that appear to be sites of end-to-end microtubule annealing (Fig. 3B, arrowheads). Because a low APM concentration produced fewer but significantly longer microtubules without a large reduction in total polymer mass (Fig. 2; Table I), long microtubules probably arose from an end-to-end annealing of short microtubules, a phenomenon described previously with animal microtubules in vitro (Rothwell et al., 1986). Apparently, annealing of tobacco microtubules occurred as a result of an APM-induced enhancement of dynamics at microtubule ends.

**High-Affinity Oryzalin Binding to Tobacco Tubulin**

The primary objective of this study was the documentation of interactions between APM and plant tubulin and microtubules. Although the unavailability of radiolabeled APM precluded direct binding measurements, information concerning APM may be deduced by examining the effects of APM on the binding of oryzalin to tobacco tubulin. Thus, we began with a characterization of [14C]oryzalin binding to tobacco tubulin.

Soluble tobacco tubulin was prepared from microtubules as described previously (Bokros et al., 1993; Hugdahl and Morejohn, 1993), and the dependence on oryzalin concentration for its binding to tubulin was examined. Pure tobacco tubulin (2 μM) was incubated with different concentrations of [14C]oryzalin (0.1-3 μM), and binding was assayed at 23°C with equilibrium dialysis, which provides quasi-equilibrium measurements (Hugdahl and Morejohn, 1993). Figure 4A displays a plot of the amount of bound oryzalin versus free oryzalin at each ligand concentration and shows saturable formation of TO complex. An initial estimate of the Kd was made with Langmuir binding isotherm analysis (Klotz, 1982; Hulme and Birdsall, 1992). The inset in Figure 4A shows a semilogarithmic plot of the concentration of free oryzalin versus the r of oryzalin and tubulin and provides a sigmoidal curve, with estimated half-maximal binding to tubulin (inflection point) at 100 nm free oryzalin.

Estimation of the Kd according to the method of Scatchard (1949) was made by plotting r/[free oryzalin] (μM−1) versus r. The plot in Figure 4B provides a line corresponding to a single affinity-class of oryzalin-binding sites (correlation coefficient = 0.96) with Kd = 117 nm and a maximum estimated molar binding stoichiometry (r) of 0.32. The results show that tobacco tubulin binds oryzalin with high affinity but that it has a relatively low oryzalin-binding capacity, with only one-third of the available dimers binding oryzalin. Decay of the oryzalin-binding site was tested by preincubating pure tubulin (1 μM) at 23°C for different times prior to performing equilibrium dialysis [14C]oryzalin binding. A plot of r versus time showed no decay of oryzalin binding for preincubation periods up to 6 h (data not shown), as observed previously with maize tubulin (Hugdahl and Morejohn, 1993).

**Competition of APM with Oryzalin for Binding to Tobacco Tubulin**

Because APM has a relatively low solubility in aqueous solutions (Fedtke, 1982) and may bind to the dialysis chamber as does oryzalin (Hugdahl and Morejohn, 1993), pilot studies were conducted on its solubility in the buffer used for ligand-binding experiments. It was determined that an APM concentration ≤110 μM remains constant for periods up to 3 h, as described in "Materials and Methods."

The concentration-dependent effects of APM on the binding of [14C]oryzalin to pure tobacco tubulin were examined with equilibrium dialysis. Tobacco tubulin (2 μM) was dialyzed for 3 h at 23°C with different concentrations of [14C]oryzalin (0.2-2 μM) in the absence or presence of different concentrations of APM (20-60 μM), and quasi-equilibrium binding measurements were analyzed on a Lineweaver-Burk plot. Figure 5 displays the plot revealing an APM concentration-dependent inhibition of oryzalin binding to tobacco tubulin. The convergence of the four derived lines on the y intercept indicates a pattern of competitive inhibition of oryzalin binding by APM. A replot of the slopes of the Lineweaver-Burk plot provides an apparent Kd of 5 μM (Fig. 5, inset). The analysis demonstrated that APM and oryzalin bind to the same site on the tobacco tubulin dimer, suggesting that plant tubulin binds APM to form a TA complex.
Figure 3. Electron micrographs of negatively stained taxol-stabilized microtubules after treatment with APM. Samples are those treated with 2% (v/v) DMSO (control) (A), 6 μM APM (B), and 15 μM APM (C). Arrowheads indicate discontinuities in microtubule lattice. Bar = 0.5 μm.
Readily Reversible APM Binding to Tobacco Tubulin

The binding results above provided circumstantial evidence of a TA complex, but it was not clear whether the putative TA was a tight complex like the TC complex (Morejohn et al., 1987b), or whether it dissociated readily into unliganded TA was a tight complex like the TC complex (Morejohn et al., 1987b), or whether it dissociated readily into unliganded TA was a tight complex like the TC complex (Morejohn et al., 1987b), or whether it dissociated readily into unliganded TA was a tight complex like the TC complex (Morejohn et al., 1987b), or whether it dissociated readily into unliganded TA was a tight complex like the TC complex (Morejohn et al., 1987b), or whether it dissociated readily into unliganded

APM had no effect (experimental A and control A), indicating that TA complex dissociated during the gel filtration, which yielded free tubulin. Thus, APM binding to tobacco tubulin is readily reversible.

Depolymerization of Microtubules in Tobacco Cells by APM and Oryzalin

To understand better the mechanism of the antimicrotubule action of APM, it was important to obtain information also concerning the APM sensitivity of microtubules in tobacco cells. The effects of APM on microtubules in tobacco

Table 1. Effects of APM on preformed, taxol-stabilized tobacco microtubule length

Electron microscope negatives of negatively stained polymer samples were used to measure microtubule length.

<table>
<thead>
<tr>
<th>APM Concentration (µM)</th>
<th>Microtubule Length (µM ± sd) (n)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.4 ± 0.7 (50)</td>
</tr>
<tr>
<td>4.0</td>
<td>3.8 ± 1.6 (33)</td>
</tr>
<tr>
<td>6.0</td>
<td>4.8 ± 3.2 (73)</td>
</tr>
<tr>
<td>10.0</td>
<td>3.5 ± 2.0 (31)</td>
</tr>
<tr>
<td>12.5</td>
<td>3.8 ± 2.0 (61)</td>
</tr>
<tr>
<td>15.0</td>
<td>4.6 ± 1.9 (72)</td>
</tr>
</tbody>
</table>

* The number of microtubules measured is given in parentheses.

Figure 4. Oryzalin concentration-dependent binding to pure tobacco tubulin. A, Saturation binding of oryzalin to pure tobacco tubulin. The mean of triplicate binding assays at each concentration of oryzalin (0.1-3 µM) was used to determine the concentrations of bound oryzalin ([Orybound]) and free oryzalin ([Oryfree]). The line was drawn using the hyperbolic curve-fitting equation $y = \frac{(a \cdot x^2)}{(b + x^2)}$. The inset shows the Langmuir binding isotherm of TO formation at 23°C (Klotz, 1982; Hulme and Birdsall, 1992). Values of $r$ are plotted versus the concentration of free oryzalin ([Oryfree]), and the arrow denotes the estimated concentration of oryzalin (0.1 µM) that coincides with the inflection point of the sigmoidal curve. The line was drawn using the sigmoidal curve-fitting equation $y = \frac{[a \cdot x]^2}{(b + x^2)} + c$. B, Apparent $K_d$ for oryzalin binding to pure tobacco tubulin. Binding data were analyzed by plotting $r/[Oryfree] (\mu M^{-1})$ versus $r$ according to Scatchard (1949). The slope of the linear regression (correlation coefficient = 0.96) represents the negative reciprocal of the apparent $K_d$ of 117 nm, and extrapolation of the line to the x intercept provides an estimated maximum molar binding stoichiometry (n) of 0.32.
Amiprophos-Methyl Binding to Tobacco Tubulin

Figure 5. Competitive inhibition by APM of oryzalin binding to pure tobacco tubulin. A, Lineweaver-Burk plot of quasi-equilibrium binding data from [14C]oryzalin binding to pure tobacco tubulin in the absence (○) and presence of 20 μM (■), 40 μM (■), and 60 μM (□) APM. All values are the means from triplicate assays. The inset shows a replot of the slopes of the Lineweaver-Burk plot versus the APM concentration and affords \( K_i = 5.0 \) μM (correlation coefficient = 0.99).

Table II. Effect of gel filtration chromatography on the dissociation of TA complex

<table>
<thead>
<tr>
<th>Tubulin Sample</th>
<th>Preincubated with APM</th>
<th>Supplemented with APM</th>
<th>Tubulin Polymerized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A</td>
<td>No</td>
<td>No</td>
<td>95</td>
</tr>
<tr>
<td>Control B</td>
<td>No</td>
<td>Yes</td>
<td>0</td>
</tr>
<tr>
<td>Experimental A</td>
<td>Yes</td>
<td>No</td>
<td>97</td>
</tr>
<tr>
<td>Experimental B</td>
<td>Yes</td>
<td>Yes</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 6. Depolymerization of microtubules in tobacco cells by APM and oryzalin. Microtubules were visualized with anti-tubulin antibody and indirect immunofluorescence microscopy, and DNA was stained with DAPI in the same cells of control (A and B), 1 μM APM-treated (C and D), and 100 nM oryzalin-treated (E and F) tobacco cultures. Bars = 15 μm.

cells were examined by indirect immunofluorescence microscopy, and the results were compared to those obtained with oryzalin. Different concentrations of APM and oryzalin (0.01–1 μM) were added to exponentially growing suspension cultures of tobacco, and both control and herbicide-treated samples were taken at different times (1–14 h) and fixed for microscopy. Microtubules were visualized with indirect immunofluorescence microscopy (Simmonds et al., 1985), and chromatin was stained with DAPI in the same cells.

Figure 6A shows abundant microtubules in a typical control interphase cell, with bright staining of microtubules around the nucleus (Fig. 6B). All microtubule arrays in cells treated with 10 and 100 nM APM were present, even after 14 h of treatment, although preprophase bands appeared wider and mitotic spindles were shorter than in control cells. At these low APM concentrations, phragmoplasts formed, normal chromosome distributions during all stages of mitosis were seen, and no polyploid or micronuclei were observed, even at later times.

In cultures treated with 1 μM APM, microtubules in many cells were depolymerized within 1 h, and very few cells retained a normal-appearing microtubule cytoskeleton. After 2 h of treatment, most cells had only short residual microtubules. At later times many cells accumulated in prometaphase and displayed chromosomes arranged in a “ball metaphase,” indicating incomplete chromosome congression to the metaphase plane. Figure 6C shows an interphase cell (top) and a mitotic cell (bottom) after treatment with 1 μM APM. The interphase cell has some short residual microtubules surrounding the nucleus (Fig. 6D), and the mitotic cell has punctate staining corresponding to short tufts of kinetochore microtubules attached to chromosomes arranged in a ball prometaphase (Fig. 6D). No normal metaphase, anaphase, or telophase cells were seen, and mitotic inhibition resulted in the formation of partial phragmoplasts, with G1 cells having restitution nuclei or a few aneuploid micronuclei.

Cells treated with 5 μM had no discernible microtubules in the interphase cell (Fig. 6E); the limited number of samples of chromosome...
Figure 7. Inhibition of tobacco cell mitosis by APM. DNA is stained with DAPI in control cells (A) and 5 μM APM-treated tobacco cells (B). Control cell nuclei and chromosomes are labeled according to stage in the cell cycle or mitosis as follows: G1, S/G2, metaphase (m), and telophase/cytokinesis (t/c). APM-treated cells are labeled as prometaphase (pm), scattered chromosomes (sc), and restitution nuclei (rn). Bar = 15 μm.

arrangements and nuclear morphology seen in DAPI-stained preparations of control cells (Fig. 7A) and cells treated with 5 μM APM (Fig. 7B). In APM-treated cells chromosomes had no kinetochore microtubules, and scattered chromosomes indicated a complete inhibition of prometaphase chromosome congression. No normal metaphase, anaphase, or telophase figures were observed, and at later treatment times chromosomes decondensed to form irregularly shaped restitution nuclei or various numbers of aneuploid micronuclei. In summary, the above results show that plant mitosis is inhibited by APM concentrations that cause the loss of microtubules. The threshold concentration \( t \) range of APM causing microtubule depolymerization and mitotic inhibition is 0.1 μM < \( t \) ≤ 1 μM.

Tobacco cells treated with 10 nM oryzalin had shorter spindles than control cells, but other microtubule arrays appeared normal, and cells divided normally. In cells treated with 50 nM oryzalin, many interphase microtubules and mitotic spindles were absent or shorter than in control cells. Although preprophase bands appeared wider, phragmoplasts formed and cells divided apparently normally without generating micronuclei. In cells treated with ≥100 nM oryzalin, most interphase microtubules were depolymerized within 1 h. Figure 6E shows a few remaining microtubules in an interphase cell in the vicinity of the nucleus (Fig. 6F) after treatment with 100 nM oryzalin. Chromosomes in 100 nM oryzalin-treated cells accumulated at prometaphase, only fragments of phragmoplasts developed, and G1 cells usually formed various numbers of irregularly sized micronuclei. Thus, as seen with APM above, oryzalin concentrations causing the loss of microtubules in cells also inhibit mitosis. The threshold oryzalin concentration range for microtubule depolymerization and mitotic inhibition is 0.1 μM < \( t \) ≤ 100 nM.

Effects of APM on Growth of Tobacco Cells

The effects of APM on tobacco cell growth in suspension culture were examined. Day seven stationary phase cells (2 mL) were transferred to medium (100 mL) in siliconized flasks containing different concentrations of APM (50 and 500 nM) and were grown as described previously (Bokros et al., 1993). Both the control and APM-containing flasks contained a final concentration of 1% (v/v) DMSO. At 1-d intervals triplicate samples (0.5 mL) of cells were sedimented by brief centrifugation in tared microfuge tubes, and fresh weights were determined. The results presented in Figure 8 show that following a 2-d lag phase, control cells grew rapidly for 4 d and entered stationary phase by d 6. Tobacco cells grown with 50 nM APM showed growth kinetics similar to those of the control culture. However, the fresh weights of these cultures were slightly greater, in general, than the controls during the stationary phase, suggesting a promotion of growth with 50 nM APM. Cells grown with 500 nM APM exhibited slow increases in fresh weight, attaining only 58% of the control fresh weight by d 9 (Fig. 8). The data demonstrate tobacco cell growth to be inhibited near the threshold APM concentration, causing the loss of microtubules and inhibiting mitosis, as described above. We are investigating how tobacco cell growth may have been enhanced by low APM concentrations.

Resistance of Mammalian Cell Microtubules to APM

Although there have been no comprehensive studies reported regarding the effects of phosphoric amides on microtubules in mammalian cells, we previously observed little or no effect of APM on microtubules in cultured PtK2 cells, a fibroblast line derived from the kidney of the marsupial rat kangaroo (Morejohn and Fosket, 1984b). To assess the potential antimicrotubule action of APM in cells of an advanced mammal, mouse 3T3 fibroblasts were treated with 100 μM APM (0.5% [v/v] DMSO) in culture medium at 37°C. Cultures were treated also with 0.5% (v/v) DMSO alone (negative control) or 400 nM colchicine (positive control). At
Amiprophos-Methyl Binding to Tobacco Tubulin

with 0.4 colchicine. Bar = 15 \mu m.

Effects of APM on microtubules in mouse 3T3 fibroblasts.

Amiprophos-Methyl Binding to Tobacco Tubulin

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Figure 9. Effects of APM on microtubules in mouse 3T3 fibroblasts. Microtubules in drug-treated cells were visualized with indirect immunofluorescence microscopy using anti-tubulin antibody. A. Cells treated with 100 \mu M APM in 0.5% (v/v) DMSO. B. Cells treated with 0.4 \mu M colchicine. Bar = 15 \mu m.
occurred as a result of the high viscosity and concomitantly lower rates of diffusion of herbicide, dimer, and polymer.

Our in vitro observations of APM-induced microtubule length redistribution indicate that the primary molecular mechanism of APM, and possibly oryzalin, is different from those of antimicrotubule drugs active against mammalian microtubules and mitosis (Wilson and Jordan, 1993). Studies on microtubule-associated protein-stabilized mammalian microtubules undergoing treadmilling have shown that the addition of low concentrations of colchicine or vinblastine results in drug incorporation into the net assembly ends of microtubules and a dramatic decrease in the addition of unliganded tubulin (Jordan and Wilson, 1990; Skoufias and Wilson, 1992). Kinetic stabilization of assembly ends occurs with little or no change in the total mass of polymer or in the lengths of microtubules (Jordan and Wilson, 1990; Skoufias and Wilson, 1992). Although we did not examine directly the kinetics at ends of APM-treated tobacco microtubules, we found that the addition of a low concentration of APM to taxol-stabilized microtubules had no great effect on polymer mass but produced an apparent annealing of microtubules. Taxol-stabilized microtubules have slow treadmilling rates because taxol decreases the magnitude of the dissociation rate constants at both microtubule ends (Kumar, 1981; Caplow and Zeeberg, 1982; Wilson et al., 1985), and they have concomitantly low annealing rates (Rothwell et al., 1986). Because the rate of microtubule annealing is greater, with microtubules having more dynamic ends (Rothwell et al., 1986), annealing of tobacco microtubules probably results from an APM-induced increase in the kinetic activities of taxol-stabilized microtubule ends. An interesting possibility is that, if APM increases the magnitude of dissociation rate constants of both ends of taxol-stabilized microtubules, the net effect might be to increase the treadmilling rate through taxol-stabilized microtubules. Studies of the rates of tubulin exchange at plant microtubule ends will be necessary to test these possibilities.

The observations of herbicide effects on microtubules in tobacco also indicate that APM and oryzalin interact with plant microtubules differently from microtubules in mammalian cells. In mammalian cells, low concentrations of antimicrotubule drugs such as vinblastine and nocodazole change the organization of the mitotic spindle and block mitosis in metaphase without causing substantial depolymerization of spindle microtubules and, thus, act primarily by kinetic stabilization of spindle microtubules (Jordan et al., 1992). Although we found that tobacco cells treated with low concentrations of APM (10 and 100 nm) or oryzalin (10 nm) had shorter spindles than control cells, cultures were not arrested in metaphase. Mitotic inhibition in tobacco cells occurred within the threshold range of APM concentrations causing the loss of microtubules. This result further suggests that low concentrations of APM destabilize the kinetics of microtubule ends rather than stabilize them. It is interesting to speculate that the promotion of tobacco cell growth by 50 nm APM results from an enhancement of microtubule kinetics, which produces an acceleration of mitosis or cytokinesis. We are studying this possibility with populations of synchronized cells.

Although our work demonstrates that APM and oryzalin bind to the same site on plant tubulin, the location of the APM/oryzalin-binding site on tubulin is not known. Information concerning the chemical nature of the APM/oryzalin-binding site may be deduced, at least in part, from the different solubility characteristics and binding affinities of APM and oryzalin. In the buffer used for our ligand-binding studies, the maximum solubilities of APM and oryzalin were 110 and 3 µm, respectively, showing an approximate 37-fold higher solubility of APM than oryzalin (Hugdahl and Morejohn, 1993). A comparison of the derived values of $K_d$ (5 µm) for APM and $K_d$ (117 nm) for oryzalin binding to tobacco tubulin indicates a 43-fold lower affinity of tobacco tubulin for APM than for oryzalin. Both APM and oryzalin are much more hydrophobic than colchicine and have much higher affinities for plant tubulin than does colchicine ($K_d = 1.03$ µm) (Morejohn et al., 1987b). Apparently, APM and oryzalin bind to a relatively hydrophobic site on the plant tubulin dimer, and the similar magnitudes of these solubility and affinity differences suggest that binding affinity increases with increased hydrophobicity. It will be interesting in future studies to determine whether colchicine binds to the APM/oryzalin site or to a different site on plant tubulin. Because we found no decay of the oryzalin-binding site after preincubation at 23°C for up to 6 h, the relatively low molar binding stoichiometries for tobacco ($r = 0.32$) and maize ($r = 0.5$) tubulins indicate pharmacological heterogeneity among plant tubulin dimers. Such heterogeneity may be derived from differences between the α or β subunit isotypes encoded by small multigene families (Fosket and Morejohn, 1992), an idea that should be addressed.

Although tubulin is generally considered to be a highly conserved protein, there are numerous differences in the primary amino acid sequences of plant and mammalian tubulins, with most nonconservative substitutions in α-tubulin (Morejohn, 1991; Fosket and Morejohn, 1992). Because plant α-tubulins also have unusual electrophoretic, immunological and peptide-mapping characteristics (Morejohn and Fosket, 1982, 1986; Morejohn et al., 1984; Fosket and Morejohn, 1992), the structure of α-tubulin alone could account for the different and kingdom-specific pharmacological properties of plant tubulins. We speculate that different plant α-tubulin isotypes are pharmacologically distinct from one another.

Neither APM nor oryzalin is an effective antimicrotubule drug in animal systems. Studies using pure bovine brain tubulin have reported the absence of oryzalin binding (Morejohn et al., 1987a; Hugdahl and Morejohn, 1993) and no effect of APM or oryzalin on microtubule assembly in vitro (Morejohn and Fosket, 1984b; Morejohn et al., 1987a). Oryzalin has no effect on microtubules in cultured cells of frog (Xenopus laevis) (Bajer and Mole'-Bajer, 1986) or of mouse (Hugdahl and Morejohn, 1993). APM was reported to have no effect on cellular microtubules in cells of frog (Bajer and Mole'-Bajer, 1986) or of a marsupial (Morejohn and Fosket, 1984b). Our current results with mouse 3T3 fibroblasts show that APM has no discernible effect on microtubules in cells of an advanced mammal. Taken together, these observations demonstrate that animal tubulins do not bind APM or oryzalin and that microtubules in plants and animals are pharmacologically distinct.

In summary, a consideration of the present ligand-binding
data and cellular observations provides information concerning the mechanism of antimitotubule herbicide action. The determined value of $K_0$ for oryzalin binding is the herbicide concentration saturating half of the available tobacco tubulin dimers and the determined $K_i$ value for APM competitive inhibition of oryzalin binding is the APM concentration at which half of the oryzalin-binding sites are occupied by APM (Hulme and Birdsall, 1992; Hugdahl and Morejohn, 1993). Thus, the value of $K_i$ for APM is predicted to be equal to or greater than the undetermined $K_0$ for APM binding to tobacco tubulin. Because the threshold concentrations of both APM (0.1 μM < $t$ ≤ 1 μM) and oryzalin (50 nM < $t$ ≤ 100 nM) causing microtubule depolymerization in tobacco cells were lower than their values of $K_i$ (5 μM) and $K_0$ (117 nM), respectively, both herbicides must act to depolymerize microtubules in cells by similar substoichiometric binding mechanisms.

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LITERATURE CITED

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