Dicoumarol: A Unique Microtubule Stabilizing Natural Product that Is Synergistic with Taxol

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ABSTRACT

In studies on the antiproliferative actions of coumarin compounds, we discovered that dicoumarol (a coumarin anticoagulant; 3,3′-methylenebis[4-hydroxycoumarin]) inhibits the first cleavage of Strongylocentrotus purpuratus (sea urchin) embryos in a concentration-dependent manner with 50% inhibition occurring at a concentration of 10 μM. Because first cleavage in sea urchin embryos is highly selective for microtubule-targeted agents, we thought that the active compounds might inhibit cell division by interacting with tubulin or microtubules. We found that dicoumarol binds to bovine brain tubulin with a Kd of 22 μM and that 0.1 μM dicoumarol strongly stabilizes the growing and shortening dynamics at the plus ends of the microtubules in vitro. Dicoumarol reduces the rate and extent of shortening, it increases the percentage of time the microtubules spend in an attenuated (paused) state, and it reduces the overall dynamics of the microtubules. The antimitotic effects of the widely used cancer chemotherapeutic agent Taxol (paclitaxel) are also mediated by suppressing microtubule dynamics. We demonstrate that exposure to combinations of Taxol and dicoumarol results in a synergistic inhibition of cell division of sea urchin embryos. The results suggest that the antiproliferative mechanism of action of dicoumarol and possibly related pharmacophores may be mediated by tubulin binding and the stabilization of spindle microtubule dynamics. Because of its low toxicity and simple chemical structure, there is potential interest to explore combinations of antimitotic coumarins with other chemotherapeutic agents to improve efficacy and lower toxicity.

INTRODUCTION

Dicoumarol, a natural anticoagulant drug chemically designated as 3,3′-methylenebis[4-hydroxycoumarin], is metabolized from coumarin in the sweet clover (Melilotus alba and Melilotus officinalis) by molds, such as Penicillium nigricans and Penicillium jensi. Coumarin (1,2-benzopyrone), the parent molecule of dicoumarol, is the simplest compound of a large class of naturally occurring phenolic substances made of fused benzene and pyrone rings (1). Our investigation of coumarin compounds originated when translated Persian literature 3 revealed that a wide spectrum of medicinal plant extracts that were in use as early as 1000 A.D. contains a high content of coumarins. 4 Subsequent analysis of scientific literature revealed numerous reports on the antiproliferative and antitumor activities of a variety of coumarin compounds, e.g., both coumarin itself and 7-hydroxycoumarin have been reported to inhibit the proliferation of a number of human malignant cell lines in vitro (2–6) and have demonstrated activity against several types of animal tumors (7–11). These compounds have also been reported in clinical trials to demonstrate activity against prostate cancer, malignant melanoma, and metastatic renal cell carcinoma (12–14).

In addition, the coumarin anticoagulants, dicoumarol (Dicumarol) and its synthetic derivative warfarin sodium (Coumadin), have been shown to decrease metastases in experimental animals (15). Warfarin sodium, largely replacing dicoumarol therapeutically as an anticoagulant, has been used for the treatment of a variety of cancers and shown to improve tumor response rates and survival in patients with several types of cancer (16–22). However, despite numerous studies, little information has been acquired on the cellular mechanism of action of coumarin compounds in the treatment of malignancies. Possibly for this reason, the coumarin compounds have not received much attention for the treatment of cancer.

On further investigation of the coumarin literature, we developed the hypothesis that coumarin compounds might inhibit cell proliferation by interfering with mitotic spindle microtubule function. Earlier studies revealed that coumarin, 7-hydroxycoumarin, and 4-hydroxycoumarin inhibit mitosis in Allium cepa root tips (23, 24). Interestingly, 7-hydroxycoumarin disorganized the mitotic spindle microtubules in A. cepa cells, leading to the random distribution of the chromosomes at metaphase (24). This is a form of cytotoxicity common to mitotic spindle poisons that inhibit mitosis by modifying microtubule dynamics (25).

Microtubules are intrinsically dynamic polymers composed of the heterodimeric protein tubulin. Both in vitro and in cells, microtubule ends switch between growing and shortening states, a behavior termed dynamic instability (see Ref. 25 for a review). Microtubule dynamics are essential for the proper movements of chromosomes during mitosis and play a crucial role in passage through the metaphase/ anaphase checkpoint. At the onset of mitosis, the interphase microtubule network is replaced by a population of spindle microtubules that are 10–100 times more dynamic than the interphase network (26). These rapid dynamics are crucial for attachment at the kinetochores and proper chromosome alignment for segregation at anaphase (25, 27). Cells with improper spindle assembly or chromosome alignment are prevented from transitioning from metaphase to anaphase by mitotic checkpoint mechanisms, resulting in cell cycle arrest in mitosis followed by apoptosis (28–30).

In the present study, we investigated the antimitotic actions of several coumarin compounds on the first division of sea urchin embryos. The first few cell divisions of sea urchin embryos exhibit a remarkable degree of pharmacological selectivity toward mitotic spindle poisons (31). We found that several coumarin compounds inhibited the first cleavage of Strongylocentrotus purpuratus embryos in a concentration-dependent manner, the most potent of the coumarins tested being dicoumarol. By video microscopy and fluorescence spectroscopy, we determined that dicoumarol binds to tubulin and suppresses the dynamic instability of individual bovine brain microtubules in vitro. In addition, we evaluated the combined antiproliferative activity of Taxol (paclitaxel) and dicoumarol in the sea urchin cell division model and found that the combination did produce synergistic inhibition of cell division. In view of the relative simplicity of the coumarin compounds and their microtubule-targeted mechanism of action, we believe the coumarin pharmacophore may serve as an...
DICOUMAROL IS SYNERGISTIC WITH TAXOL.

MATERIALS AND METHODS

Materials. All drugs and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Inhibition of Cell Cleavage. Sea urchins, either *S. purpuratus* or *Strongylocentrotus franciscanus*, were induced to spawn by injection of 0.5 M KCl into the coelomic cavity and cultured in filtered seawater, as described previously (31). Eggs were used in fresh filtered seawater at a concentration of ~1% (v/v).5 Sperm were collected directly from the aboral surface of male sea urchins and diluted into seawater (one drop of concentrated sperm/1 ml of seawater).

Eggs were fertilized by adding diluted sperm to the diluted egg suspensions (1 ml of sperm suspension/100 ml of diluted egg suspension) and then added to various drug concentrations. The embryos were then incubated at 15°C in a water bath for ~120 min after fertilization. Quantification of division was performed visually by light microscopy by counting the number of cleaved and noncleaved embryos after the control embryos had progressed to the end of first cleavage.

The effect of dicoumarol at different stages of the cell cycle was determined by adding dicoumarol at 10-min intervals after fertilization to aliquots of sea urchin embryos and scoring the inhibition percentage of first cleavage. To examine the reversibility of the compound, dicoumarol was added to embryo suspensions within 1 min of fertilization. Exposure to the compound was terminated at 10-min intervals by sedimenting and resuspending the embryos three times in fresh seawater.

Purification of MTP, Tubulin, and Flagellar Axonmental “Seeds.” Bovine brain MTP (70% tubulin and 30% microtubule-associliated proteins) was purified by three cycles of warm polymerization and cold depolymerization in vitro. Tubulin was purified from the MTP by phosphocellulose chromatography as described previously (32). The MTP and purified tubulin were quickly frozen as drops in liquid nitrogen and stored at ~70°C until used. Axonemal seeds were prepared from sea urchin sperm (*S. purpuratus*; Ref. 32). All protein concentrations were determined by the method of Bradford (33) using BSA as the standard.

Assembly of Microtubules and Determination of Steady-state Microtubule Polymer Mass. Bovine brain MTP and phosphocellulose-purified tubulin pellets were thawed and centrifuged at 4°C to remove any aggregated or denatured tubulin. Several reaction mixtures were prepared to determine the effects of dicoumarol on microtubule assembly. Tubulin (2.5 mg/ml) was polymerized in the absence or presence of a range of dicoumarol concentrations (35 min; 37°C) in PME buffer (100 mM piperezinedithanesulfonic acid, 1 mM MgCl2, 1 mM EGTA (pH 6.8), and 100 mM GTP). The same reaction mixture was used as described above, using *S. purpuratus* axoneme seeds or glycogenylated axonemal seeds for assembly initiation. To prepare glycogenylated microtubule seeds for nucleation, 30 μM tubulin was polymerized in PME buffer containing 10% glycerol (v/v) and 100 mM GTP for 30 min at 30°C. After 30 min of polymerization, microtubules were sheared by passage (~10 times) through the nozzle of a 50-μl Hamilton syringe to form seeds. Seeds were then added in a ratio of 1:5 v/v to the polymerization mixture and warmed to room temperature. The final glycerol concentration was 2%. For studies with MTP, samples containing 2.5 mg of MTP/ml were incubated in PME buffer containing 100 mM GTP in the presence or absence of various dicoumarol concentrations without seeds. All of the above reaction mixtures were polymerized for 35–45 min at 37°C to achieve near steady state, and microtubule polymerization was monitored by turbidimetry at 350 nm using a Gilford Response Spectrophotometer. To determine the microtubule mass, microtubules assembled in the above reaction mixtures were pelleted by centrifugation at 150,000 × g for 1 h at 37°C. Microtubule pellets were solubilized in PME buffer at 0°C for protein determination.

Determination of Microtubule Dynamic Instability by Video Microscopy. Tubulin (12 μM) was polymerized to steady state onto flagellar seeds in the absence or presence of dicoumarol. The seed concentration was adjusted to achieve three to six seeds per microscope field. After 35 min of incubation, samples of microtubule suspensions (2 μl) were prepared for video microscopy, and the dynamic instability behavior of individual microtubules was recorded at 37°C and analyzed as described previously (34). Microtubules were observed for a maximum of 45 min after they had reached steady state. We considered a microtubule to be in a growth phase if it increased in length by >0.2 μm at a rate >0.15 μm/min and in a shortening phase, if it shortened by >0.2 μm at a rate > 0.3 μm/min. Microtubules undergoing length changes ≤ 0.2 μm over the duration of six data points were considered to be in an attenuated state. The same tubulin preparation was used for all experiments; 20–30 microtubules were analyzed for each experimental condition.

The catastrophe frequency [a catastrophe is a transition from the growing or attenuated state to shortening (35)] was determined by dividing the number of catastrophes by the sum of the total time spent in the growing plus attenuated states for all microtubules for a particular condition. The rescue frequency [a rescue is a transition from shortening to growing or attenuation, excluding new growth from a seed (35)] was calculated by dividing the total number of rescue events by the total time spent shortening for all microtubules for a particular condition. Dynamicity is the sum of all growing and shortening events divided by the total time measured, including time spent in the attenuated state (32).

Fluorescence Spectroscopy. Fluorescence spectroscopy was performed using a Perkin-Elmer LS50B spectrofluorometer. Spectra were taken by multiple scans, and buffer blanks were subtracted from all measurements. The inner filter effects were corrected as described by Sackett (36) and empirically by measuring the change of fluorescence intensity of a tryptophan solution equivalent to the tubulin concentration in the presence of dicoumarol. Dicoumarol did not quench the fluorescence of tryptophan in solution after inner filter effect correction. The excitation and emission wavelengths were 295 and 336 nm, respectively. The fraction of binding sites (β) occupied by dicoumarol was determined using the following relationship: β = (Fo − F)/Fo = Fm, where Fo is the fluorescence intensity of tubulin in the absence of dicoumarol, F is the corrected fluorescence intensity when the tubulin and dicoumarol are in equilibrium, and Fm is the calculated fluorescence intensity of the fully liganded tubulin. Fm was determined by plotting 1/(Fo − F) versus 1/L (L = total ligand concentration) and extrapolating 1/L = 0. The association constant, Ka, was determined using the relationship: Ka = (β(1 − β)) × 1/Lf, where Lf = L − β(C) and [C] is the molar concentration of ligand-binding sites, assuming a single binding site per tubulin dimer.

Analysis of Drug Synergism. The CI was used to determine whether the drugs interacted synergistically, additively, or antagonistically (37). The CI is defined by the following equation:

\[
CI = \frac{(D_1)}{(Dx)_1} + \frac{(D_2)}{(Dx)_2}
\]

where (D1) is the concentration necessary for a particular effect in the combination, (Dx)1 is the dose of the same drug that will produce the identical level of effect by itself, (D2) is the concentration of a second drug that will produce a particular effect in the combination, and (Dx)2 is the concentration of the second drug that will produce the same level of effect by itself. For combinations, a CI > 1 implies antagonism, a CI < 1 indicates synergy, and a CI = 1 indicates additivity.

RESULTS

Inhibition of Cell Division by Dicoumarol. In preliminary studies on the antimitotic actions of coumarins, we found that several coumarin compounds inhibited the first cleavage of *S. purpuratus* embryos in a concentration-dependent manner with 50% inhibition occurring at 10 μM dicoumarol, 27 μM coumarin, 36 μM 7-hydroxy-coumarin, and 25 μM warfarin (data not shown). Dicoumarol was the most potent of the coumarin compounds examined; IC50 = 10 μM (Fig. 1) with a complete block of cell division at concentrations > 20 μM. Dicoumarol-treated embryos showed no lysis or morphological changes at any of the concentrations treated. Identical concentration-
DICOUMAROL IS SYNERGISTIC WITH TAXOL.

Visual analysis of the traces revealed that 0.1 μM dicoumarol significantly suppressed the dynamics.

The effects of dicoumarol (0.04–50 μM) on the individual dynamic instability parameters are shown quantitatively in Table 1. Dicoumarol inhibited the rate and extent of microtubule shortening in a concentration-dependent manner (Fig. 5A). The addition of 1 μM dicoumarol significantly reduced the mean shortening rate by ~58%, from 18.5 to 7.8 μm/min, and it reduced the length of a shortening excursion by ~40%, from 2.3 to 1.4 μm. In contrast to the strong action of dicoumarol on the rate and length of shortening, there was no significant change in the average rate or length of growth at the various dicoumarol concentrations examined (Fig. 5B).

Steady-state microtubules in vitro and in cells spend a portion of time in an attenuated (or paused) state (32). At concentrations as low as 0.1 μM, dicoumarol significantly increased the overall percentage of time in the attenuated state.

The effects of dicoumarol on the frequency of transition among growth or shortening at microtubule ends was not detectable.

response curves were also obtained using Lytechinus pictus and S. franciscanus embryos (data not shown).

Cell Cycle Analysis and Reversibility of Dicoumarol. To investigate where in the cell cycle dicoumarol exerts its activity, sea urchin embryos were exposed to dicoumarol at progressively later times after fertilization. This allows rapid identification of phases of the cell cycle that are sensitive to the drug. Fig. 2 shows that maximal inhibition of embryo cleavage occurred when the dicoumarol was added as late as 75 min after fertilization, well after the time of DNA synthesis. Inhibition declined steeply when dicoumarol was added 90 min after fertilization, during prophase and pro-metaphase of mitosis. These results suggest that dicoumarol inhibits cell division by blocking cells at prometaphase or metaphase of mitosis.

We examined whether inhibition of cell division by dicoumarol could be reversed. Embryos were incubated with 50 μM dicoumarol (a concentration which inhibits cell division by 100%); beginning immediately after fertilization, the embryos were washed by sedimentation and resuspended in fresh seawater to remove the compound at 10-min intervals until the time of first cleavage (2 h). As shown in Fig. 2B, the effects of dicoumarol appear to be ~30% (t = 35–65 min) to 60% (t = 15 min) reversible when the compound is removed before M phase of the cell cycle. Thus, dicoumarol is reversible until the onset of mitosis.

Binding of Dicoumarol to Tubulin. Tubulin (2 μM) was incubated with a range of dicoumarol concentrations for 30 min at 34°C in 50 mM PME buffer. Dicoumarol quenched the intrinsic fluorescence of tubulin in a concentration-dependent manner. The binding constant determined from the data was 22 μM (Fig. 3), and the dicoumarol bound to the tubulin with a 1:1 stoichiometry.

Effects of Dicoumarol on Microtubule Polymerization. As determined by sedimentation and light scattering assays, a wide range of dicoumarol concentrations (0–100 μM) incubated with PC-tubulin, axoneme-seeded PC-tubulin, glycerol-seeded PC-tubulin, or MTP did not produce any significant alteration in the polymer mass or the rate and extent of polymerization of free tubulin into microtubules (data not shown). However, because recent data suggest that the suppression of microtubule dynamics rather than the stimulation or inhibition of microtubule polymerization is the primary anticancer mechanism of action of many antimitotic drugs (see Ref. 25 for review), we examined the effects of dicoumarol on microtubule dynamic instability in vitro.

Effects of Dicoumarol on Microtubule Dynamic Instability. The effects of dicoumarol on the growth and shortening dynamics at the plus ends of individual microtubules in vitro were analyzed by video microscopy. Several life history traces of microtubule length changes with time in the presence and absence of 0.1 μM dicoumarol are shown in Fig. 4. Control microtubules alternated between phases of growing and shortening but also spent a small percentage of the time in an attenuated state, periods during which

![Figure 1](image1.png)

Fig. 1. Log concentration response curve for inhibition of S. purpuratus embryo cleavage by dicoumarol. Pooled results from eight representative experiments are shown. Error bars, SE.

![Figure 2](image2.png)

Fig. 2. A, the effect of dicoumarol at different stages of the cell cycle in S. purpuratus embryos. Dicoumarol (50 μM) was added to embryo suspensions at the times indicated after fertilization. Incubation was continued until control embryos had completed first cleavage. Pro, Met, Ana, and Tel are prophase, metaphase, anaphase, and telophase of mitosis, respectively. B, reversibility of 50 μM dicoumarol during the first division cycle. The drug was added 1-min postfertilization, then at 10-min intervals; a 1-ml sample of 1% (v/v) suspension of eggs in seawater was washed three times with 10 ml of filtered seawater.

![Figure 3](image3.png)

Fig. 3. Effects of dicoumarol on tubulin fluorescence. Tubulin (2 μM) was incubated in the absence or presence of various dicoumarol concentrations at 25°C for 30 min, and the fluorescence was determined as described in “Materials and Methods.” The above figure shows protein fluorescence quenching titration after binding of dicoumarol to tubulin. Fluorescence values at 336 nm were taken for the calculation. Data are representative of four different experiments.
phases of growing, shortening, and attenuation were calculated as events per unit of time and are considered important parameters in the regulation of microtubule dynamics in cells. They are also indicators of whether the drug is acting on the stabilizing cap. Dicoumarol decreased the catastrophe frequency at concentrations as low as 0.1 μM, by 65%, from 0.62 to 0.22 events/min. These results indicate that dicoumarol may stabilize the cap at microtubule plus ends. Dicoumarol had no apparent effect on the rescue frequency.

Dynamicity is the summed gain and loss (exchange) of tubulin subunits at the microtubule ends and is a measure of overall dynamic instability behavior. Thus, dicoumarol significantly decreases the dynamic instability behavior of microtubules, primarily by decreasing the catastrophe frequency and inhibiting the rate and extent of microtubule shortening.

**Synergistic Inhibition of Cell Division by Dicoumarol and Taxol.** To determine whether the effect of dicoumarol on division of sea urchin embryos could be additive or possibly synergistic with the chemotherapeutic agent, Taxol, we investigated the combined effects of the two compounds on cleavage. In the presence of 5 μM dicoumarol (a concentration that inhibits cell division by ~25%), the IC_{50} for Taxol was effectively lowered by >2-fold (Fig. 6A). Combinations of Taxol with dicoumarol produced a more pronounced inhibition of cell division than expected. As seen in Fig. 6B, the inhibition percentage of cell division in cells incubated with 0.3 μM Taxol increased by >70% in the presence of 5 μM dicoumarol. Calculation of the CI indicates that Taxol and dicoumarol act synergistically to inhibit cell proliferation (CI = 0.31).

**DISCUSSION**

**Mechanism of Action of Dicoumarol.** The fertilized sea urchin egg has shown utility as an experimental cell model for investigating mechanisms of drug action. Eggs fertilized at the same time undergo numerous synchronous divisions, allowing rapid identification of drug-induced inhibition of cell division. In addition, the first cell cycle exhibits a remarkable degree of pharmacological selectivity for mitotic spindle poisons while being relatively insensitive to agents acting by other common inhibitory mechanisms. In the present study we found that dicoumarol produced a concentration-dependent inhibition of the first cell division of the sea urchin embryo (IC_{50} = 10 μM).

We then examined the ability of the compound to block cells in mitosis, as would be expected of a mitotic spindle poison. The first mitosis typically occurs between 70 and 90 min after fertilization, and cytokinesis is usually complete at 120 min. By adding dicoumarol to fertilized embryos at different times before the completion of the first cleavage, we found that the ability of dicoumarol to inhibit the first cell division occurred when added as late as 75 min after fertilization. These results suggested that dicoumarol selectively inhibits cell cycle progression at prometaphase/metaphase of mitosis, and actions on any events before the beginning of M phase are not crucial to the activity of the drug.

Because dicoumarol inhibits cell division by acting on a cellular process essential during mitosis, we reasoned that the cellular target for these compounds might be tubulin or microtubules. We first analyzed the effects of dicoumarol on microtubule assembly in vitro and found that even at very high concentrations (100 μM), the drug did not produce any appreciable changes in the rate or extent of polymerization. However, because the suppression of microtubule dynamics by antimitotic drugs in the absence of appreciable change in polymer mass occurs at the lowest effective concentrations with most of these compounds, we then examined the effects of dicoumarol on

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**Table 1** Effects of dicoumarol on the dynamic instability parameters at microtubule plus ends at steady state

<table>
<thead>
<tr>
<th>Concentration, μM</th>
<th>0</th>
<th>0.04</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>50</th>
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<tbody>
<tr>
<td><strong>Mean rate (μm/min)</strong></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Growing</td>
<td>0.79 ± 0.1 *</td>
<td>0.61 ± 0.07</td>
<td>0.53 ± 0.1</td>
<td>0.63 ± 0.08</td>
<td>0.63 ± 0.1</td>
<td>0.54 ± 0.07</td>
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<tr>
<td>Shortening</td>
<td>18.5 ± 7.2</td>
<td>18.9 ± 8.1</td>
<td>12.1 ± 4.6</td>
<td>7.8 ± 4.8</td>
<td>7.4 ± 2.4</td>
<td>1.1 ± 0.2</td>
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<tr>
<td><strong>Mean length (μm/event)</strong></td>
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<tr>
<td>Growing</td>
<td>0.86 ± 0.15</td>
<td>0.70 ± 0.09</td>
<td>0.61 ± 0.1</td>
<td>0.95 ± 0.22</td>
<td>0.58 ± 0.09</td>
<td>1.0 ± 0.17</td>
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<tr>
<td>Shortening</td>
<td>2.3 ± 0.58</td>
<td>2.0 ± 0.53</td>
<td>1.9 ± 0.43</td>
<td>1.4 ± 0.44</td>
<td>1.6 ± 0.37</td>
<td>1.4 ± 0.21</td>
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<tr>
<td><strong>Mean duration (min)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Growing</td>
<td>1.7 ± 0.52</td>
<td>1.3 ± 0.18</td>
<td>1.5 ± 0.29</td>
<td>1.7 ± 0.30</td>
<td>1.7 ± 0.38</td>
<td>2.36 ± 0.34</td>
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<tr>
<td>Shortening</td>
<td>0.53 ± 0.18</td>
<td>0.37 ± 0.13</td>
<td>1.1 ± 0.43</td>
<td>0.86 ± 0.34</td>
<td>0.51 ± 0.15</td>
<td>1.4 ± 0.07</td>
</tr>
<tr>
<td><strong>Total time (%)</strong></td>
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<tr>
<td>Growing</td>
<td>59.4</td>
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<tr>
<td>Shortening</td>
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<td>22</td>
<td>27.8</td>
<td>17.3</td>
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<tr>
<td>Attenuated</td>
<td>13.7</td>
<td>17.7</td>
<td>37.6</td>
<td>50.5</td>
<td>71.2</td>
<td>51.3</td>
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<tr>
<td><strong>Transition frequencies (events/min)</strong></td>
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<tr>
<td>Catastrophe</td>
<td>0.62</td>
<td>0.7</td>
<td>0.22</td>
<td>0.24</td>
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<td>Rescue</td>
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<td>1.03</td>
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<td><strong>Dynamicty</strong></td>
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<td>1.2</td>
<td>0.65</td>
<td>0.43</td>
<td>0.24</td>
<td>0.31</td>
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* ±, SE.
microtubule dynamic instability in vitro. We found that at concentrations that did not affect the polymer mass, dicoumarol strongly suppressed microtubule dynamic instability. It suppressed the shortening rate more significantly than the growing rate and decreased the catastrophe frequency, resulting in stabilized microtubules.

Direct evidence that dicoumarol binds to tubulin was obtained by fluorescence spectroscopy. A dissociation constant of 22 μM and a binding stoichiometry of 1 mol of dicoumarol per mol of tubulin were determined by analyzing the ability of dicoumarol to quench the intrinsic fluorescence of tubulin. Dicoumarol decreased the intrinsic fluorescence of tubulin, most likely by causing a conformational change in tubulin in the region of a fluorophore, such as a tryptophan residue on binding. The conformational change in tubulin induced by dicoumarol binding may be an important factor in its ability to stabilize microtubule dynamics. It is also possible that in the presence of dicoumarol, the affinity between adjacent dimers is increased, making tubulin dissociation less favorable.

Microtubules are composed of an unstable tubulin-GDP core and a stable tubulin-GTP or tubulin-GDP-Pi “cap” at the microtubule ends. It is believed that microtubule dynamic instability is caused by the gain and loss of this stabilizing cap at microtubule ends (38). Loss of the cap is thought to be required for initiation of a shortening phase. Dicoumarol may stabilize microtubules by binding to unstable GDP-ligated tubulin with a higher affinity than growing GTP-ligated tubulin or that the conformational change induced in tubulin by dicoumarol resembles that of the stable tubulin-GTP cap. It is also possible that dicoumarol reduces the rate of GTP hydrolysis or Pi release, preventing cap loss and a decrease in the frequency of catastrophe.

Comparison of the Mechanism of Action of Dicoumarol and Taxol. Antimitotic agents that interact with microtubules are of interest because of their potential uses in the treatment of human neoplastic and inflammatory diseases. Although substoichiometric concentrations of diverse antimitotic drugs, such as Taxol, vinblastine, and colchicine, suppress microtubule dynamics in cells and inhibit cell proliferation, the mechanism by which these drugs affect dynamic instability parameters, such as growing and shortening rates, duration of attenuated states, or transition frequencies (rescues and catastrophes), differs. Dicoumarol appears to be most similar mechanistically to Taxol in its ability to stabilize microtubule dynamics. The most potent action of Taxol on dynamic instability is the suppression of the rate and extent of shortening in the absence of an appreciable effect on the rate or extent of growth (39), an effect we find is shared by dicoumarol. In addition, both compounds greatly increase the percentage of time the microtubules spend in the attenuated state. This is in contrast to the mechanism of other common microtubule-stabilizing agents, such as vinblastine and colchicine, which stabilize plus ends of the microtubules by suppressing both the rate and extent of growth and shortening (32, 34, 40). We believe that in this respect, the mechanism by which dicoumarol stabilizes microtubule dynamics is Taxol-like.

There are, however, several notable differences in the action of dicoumarol and Taxol. Taxol exhibits differential effects on microtubule properties at different binding stoichiometries. Substoichiometric binding of Taxol to tubulin in microtubules suppresses the microtubule shortening rate but does not significantly affect the growing rate or polymer mass (39). However, at Taxol concentrations that are stoichiometric with respect to tubulin concentrations in solution, there is extensive stimulation of the rate and extent of microtubule polymerization in vitro (41). It enhances nucleation and elongation phases of microtubule polymerization reaction in vitro and reduces the critical subunit concentration (39). Unlike the actions of Taxol, high concentrations of dicoumarol do not produce an appreciable change in the rate or extent of microtubule polymerization. An additional difference between dicoumarol and Taxol is their difference in tubulin-binding properties. Taxol binds preferentially to the polymeric microtubule form of tubulin and binds extremely poorly to soluble tubulin (42–44). Dicoumarol, however, binds to tubulin dimers in 1:1 stoichiometry with moderate affinity. This difference suggests that dicoumarol and Taxol may have different binding sites, which may prove useful in formulating combination therapy approaches.

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**Fig. 5.** Effects of dicoumarol on the shortening (A) and growing (B) rates of individual microtubules at their plus ends.

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**Fig. 6.** Synergy between Taxol and dicoumarol. The combination of Taxol with dicoumarol decreases the concentration of Taxol required to induce 50% inhibition of cell proliferation in the sea urchin cell division assay. The results are expressed as the percentage of inhibition compared with vehicle and the means of five separate experiments; bars, SE.
**Implications for Combination Therapy.** Evidence suggests that combination therapy with drugs that stabilize microtubule dynamics by different mechanisms may improve responses and minimize side effects of the individual drugs. Although Taxol has shown good efficacy against refractory ovarian cancer, metastatic breast cancer, head and neck cancer, melanoma, and lung cancer, its use in the clinic is hampered by its side effects, low solubility, and narrow therapeutic index (45). Recent clinical trials have indicated that combinations of antimitotic drugs result in increased antitumor activity and decreased toxicity (46–50), e.g., combinations of Taxol and estramustine, and enhance cytotoxicity against human prostate carcinoma (47, 50).

Estramustine, an antitumor drug used in the treatment of hormone-refractory prostate cancer, is similar to dicoumarol in that it weakly binds to tubulin (Kd ≈ 30 μM), does not appreciably affect the microtubule polymer mass, but strongly reduces the overall dynamism of the microtubules (51). Thus, compounds with the same intracellular target, but different binding sites and modes of action, may have the potential to be used in combination for chemotherapeutic purposes.

We have demonstrated that the combination of both drugs acts synergistically to the inhibit cell division in sea urchin embryos. This observation is similar to that obtained in a study in which the combination of Taxol and coumarin on meristematic cells of Allium sativum root tips increased antimitotic activity and was associated with fewer cytotoxic and clastogenic effects than when Taxol was used alone (52). Together, these results suggest that dicoumarol and Taxol have the potential to be used together to improve efficacy and lower toxicity.

It is also possible that the coumarin anticoagulants in combination with Taxol may improve cancer survival rates by other mechanisms. Tumor cell invasion is dependent on angiogenesis and requires both cell migration and digestion of basement membrane by proteases (53). Anticoagulants were thought to reduce the incidence of cancer metastases by inhibiting formation of a fibrin matrix required for the fixation of circulating cancer cells. Because Taxol has been reported to inhibit migration of human ovarian and prostate carcinoma cells (54, 55) and the coumarin anticoagulants inhibit the thrombin-induced release of matrix metalloproteinases that cause the breakdown of extracellular matrix proteins (56), it is logical to assume that dicoumarol might enhance the antiangiogenic activity of taxanes. Coumarin anticoagulants inhibit the pathway involving tissue factor and factor VIIa. Tissue factor VIIa appears to be a major factor in the regulation of angiogenic growth properties of tumor cells, and in vitro studies have demonstrated a significant role for warfarin in the regulation of the inhibition of angiogenesis (57). Taxol also has been shown to display antiangiogenic properties in association with the down-regulation of vascular endothelial growth factor, a pro-angiogenic factor that can act directly on endothelial cells to promote vessel formation (58). Because these two compounds possess the ability to inhibit angiogenesis by different mechanisms, it is possible that when combined, they can produce a synergistic effect on the inhibition of tumor growth by a mechanism other than stabilization of microtubule dynamic instability. It is however important to stress that although coumarin comprises the substructure of dicoumarol and warfarin, it does not contain any anticoagulant properties. It would be of great interest to further investigate the potential antiangiogenic effect of coumarin anticoagulants and Taxol combinations in vitro and in vivo.

In summary, our results indicate that dicoumarol may exert its antiproliferative effects by stabilizing spindle microtubule dynamics through a unique interaction with tubulin and microtubules. The structures of the known antimitotic chemotherapeutic drugs, such as the taxanes and Vinca alkaloids, are complex and not easily manipulated chemically. Dicoumarol and coumarins could provide a new structural class for synthetic elaboration that could lead to improved antineoplastic drugs. The simple chemical structure of the coumarins allows great potential to clinically explore combinations of coumarin analogs with other microtubule-stabilizing agents in an attempt to improve efficacy. In addition, dicoumarol and possibly other coumarin compounds that act by stabilizing microtubule dynamics might have entirely new tumor specificity than the currently used antimitotic agents. Their synergistic activity in combination therapy could be the basis for development of rational approaches to new forms of cancer chemotherapy.

**ACKNOWLEDGMENTS**

We thank Seyedeh Nasnir Sohrab for access to important Persian medica medicus in developing conceptual aspects underlying this research. We also thank Herb Miller for excellent technical assistance and Mary Ann Jordan for advice and comments through the course of this work.

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Hamta Madari, Dulal Panda, Leslie Wilson, et al.


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