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Demonstration of microtubule-like structures formed with (−)-rhazinilam from purified tubulin outside of cells and a simple tubulin-based assay for evaluation of analog activity

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ABSTRACT

(−)-Rhazinilam was spontaneously generated from a natural product during isolation. In cultured cells, it causes microtubule bundle formation, like those caused by paclitaxel. With tubulin, (−)-rhazinilam causes formation of an aberrant spiral polymer. Using glutamate and GTP, we developed an assay for spiral formation and applied it to 17 new (±)-rhazinilam analogs with either a modified side chain or a different size D ring. There was reasonable correlation between spiral formation and inhibition of human MCF-7 breast carcinoma cell growth. Only one side chain analog was as active as (±)-rhazinilam. During these studies, we observed that omitting GTP from the reaction mixture caused a major change in the morphology of the (−)-rhazinilam-induced polymer, with half the observed polymer being microtubule-like and half being spirals. This mixed polymer slowly disassembled at 0 °C, but there was no apparent difference in the lability of the microtubules versus the spirals.

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Of all the antimitotic compounds that interact with tubulin, one of the most unusual is (−)-rhazinilam (**1**). Obtained from a number of plant species of the *Apocynaceae* family, (−)-rhazinilam was found not to be a true natural product. Instead, the actual natural product (**2**) rapidly degrades to the biologically active (−)-rhazinilam [1,2]. Structures of **1** and **2** are shown in Fig. 1.

The biological activity of (−)-rhazinilam is as unusual as its origin. Although relatively weak as a cytotoxin for an antitubulin agent, since typically it arrests cell growth with IC_{50}'s[1] in the low μM range, (−)-rhazinilam has effects on cells most similar to those of paclitaxel. It causes mitotic arrest and formation of extensively bundled, short microtubules in interphase cells [3]. With microtubule protein[2] or purified tubulin, however, (−)-rhazinilam acts more like the vinca alkaloids [4] or the colchicine site drug curacin A [5]. In some studies (−)-rhazinilam inhibits tubulin polymerization [2], while at higher concentrations it causes formation of a morphologically aberrant spiral polymer (sometimes called an "aggregation" reaction) [3]. Paclitaxel, in contrast, causes the hyperassembly of tubulin and microtubule protein into polymers of reasonably normal morphology, as well as tubulin hyperassembly in cells with formation of bundled microtubules [6,7].

Our studies with rhazinilam began with chemical synthetic work to gain greater SAR understanding of the compound, with the goal of designing a more active analog. There have been a number of previous synthetic efforts aimed at producing more active analogs of rhazinilam, but none has succeeded thus far in produc-

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[1] *Abbreviations used:* IC_{50}, the concentration of a compound that causes 50% inhibition of cell growth or of tubulin assembly; EC_{50}, the concentration of a compound that causes removal of 50% of the tubulin in the reaction mixture by centrifugation.

[2] Microtubule protein is the term commonly used to indicate a tubulin preparation prepared from a tissue extract, generally brain tissue, by cycles of assembly and disassembly. Such preparations generally contain 70–80% tubulin plus a variety of additional proteins referred to as microtubule-associated proteins. In this paper we will use the terms "tubulin" and "purified tubulin" interchangeably, referring to a protein preparation from which the microtubule-associated proteins have been removed.

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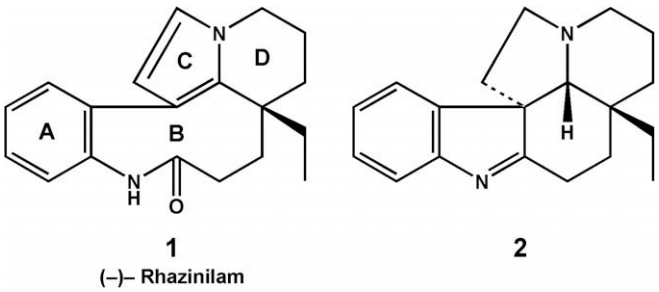

Fig. 1. Structures of (−)-rhazinilam (**1**) and its presumptive precursor (**2**).

ing a compound significantly more cytotoxic than the parent compound [8]. We directed our attention to two features of the rhazinilam molecule, ring D and the side chain attached to the junction of rings B and D (ring nomenclature as in David et al. [9]).

Quantitation of inhibitory compound effects on tubulin assembly is straightforward, and usually turbidimetric analysis of assembly data is used [10]. We initially attempted to apply our standard method to evaluate the inhibitory phase of (−)-rhazinilam's effects on tubulin assembly, but we found that the inhibitory window was too narrow, particularly with many of our newly synthesized analogs. We therefore decided to explore development of a centrifugal method to quantitate (−)-rhazinilam and analog effects on formation of the spiral polymer. We found that spiral formation was so extensive that we could readily determine compound concentrations that caused 50% of the tubulin to form these aberrant polymers.

In evaluating a number of possible reaction conditions, we also examined polymer morphology in the electron microscope. With the reaction condition chosen for quantitative evaluation of analog effects, the polymer induced by (−)-rhazinilam consisted entirely of tubulin spirals. However, in the course of these studies we found that if no GTP were added to the reaction mixture, a mixture of spirals and microtubule-like polymers was formed. As far as we are aware, this represents the first observation of polymer of "normal" morphology induced by (−)-rhazinilam in a biochemical assay with pure tubulin.

Materials and methods

Materials

Synthetic (−)-rhazinilam was a generous gift of Dr. F. Guéritte, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France. The synthesis of the rhazinilam analogs ((±)-rhazinilam and diastereoisomeric compounds **3**–**19**) will be described elsewhere (manuscript in preparation). Purified bovine brain tubulin was prepared as described previously [11]. GTP, from Sigma, was repurified by triethylammonium bicarbonate anion exchange chromatography and was over 99.5% pure at the time of isolation (periodic reevaluation has shown no significant deterioration).

Tubulin assembly assay

Reaction mixtures contained 10 μM (1.0 mg mL^{-1}) tubulin, 0.75 or 0.80 M monosodium glutamate (pH of 2.0 M stock solution adjusted to pH 6.6 with HCl), 4% (v/v) dimethyl sulfoxide as compound solvent, and concentrations of GTP and individual compounds as indicated.

In the centrifugal assay, sample incubation was for 20 min at 30 °C, followed by centrifugation for 15 min at 14,000 rpm at room temperature (about 20 °C) in an Eppendorf benchtop centrifuge. Aliquots of the supernatants were removed and their protein content determined by the Lowry method. Aliquots of uncentrifuged reaction mixtures were also evaluated for total protein content of reaction mixtures. The EC_{50} was defined as the concentration of agent required to remove 50% of the tubulin from the supernatant versus reaction mixtures without compound.

Reactions were also followed turbidimetrically in Beckman DU7400/7500 spectrophotometers, as described in detail elsewhere [10].

Electron microscopy

Aliquots of reaction mixtures were placed on 200-mesh carbon-coated, Formvar-treated copper grids and immediately stained with 5–10 successively applied drops of 1% (w/v) uranyl acetate. Excess stain was wicked from the grids with torn filter paper. The grids were examined in a Zeiss model 10CA electron microscope.

Growth of human breast carcinoma MCF-7 cells

Cells, obtained from the National Cancer Institute drug screening group, were grown in RPMI-1600 supplemented with 10% heat-treated fetal bovine serum. Cell growth was measured by quantitating cellular protein with sulforhodamine B [12]. About 5000 cells per well were seeded onto a 96-well microtitre plate and allowed to adhere overnight. Drug effects on growth were determined following an additional 48 h incubation at 37 °C in a humidified atmosphere containing 5% CO_2. The final dimethyl sulfoxide concentration was 0.5% (v/v).

Results

Initial evaluation of effects of (−)-rhazinilam on tubulin assembly in 0.8 M glutamate

We have used glutamate for quantitative evaluation of inhibitors of tubulin assembly [10], including drugs such as vinblastine, dolastatin 10, and curacin A that cause various aberrant assembly reactions [4,5,13]. An initial study (Fig. 2) suggested that this method would be effective with (−)-rhazinilam as well, but the narrow window between the end of the inhibitory effect (3 μM in Fig. 2, curve 4) and the onset of spiral induction (4 μM in Fig. 2, curve 5) proved too narrow to obtain consistent results, particularly with many of the analogs we examined.

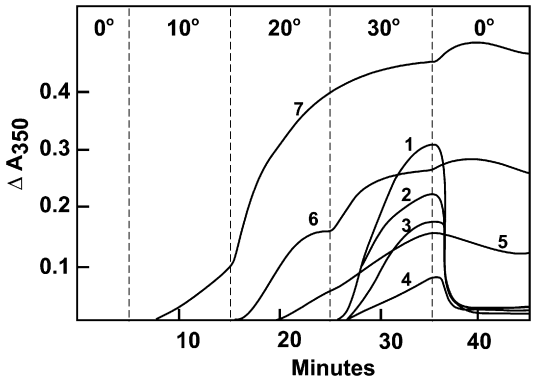

Fig. 2. Effects of varying concentrations of (−)-rhazinilam on glutamate-induced assembly with 0.4 mM GTP. Each reaction mixture contained 10 μM (1.0 mg mL^{-1}) tubulin, 0.8 M monosodium glutamate, 0.4 mM GTP, 4% dimethyl sulfoxide, and concentrations of (−)-rhazinilam as follows: curve 1, none; curve 2, 1.0 μM; curve 3, 2.0 μM; curve 4, 3.0 μM; curve 5, 4.0 μM; curve 6, 5.0 μM; curve 7, 10 μM. The temperature controller was set to the indicated temperatures at the times indicated by the vertical dashed lines to the left of each temperature. In this experiment there was a tubulin-drug preincubation without GTP for 15 min at 30 °C [10].