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CORNIGERINE, A POTENT ANTIMITOTIC *COLCHICUM* ALKALOID OF UNUSUAL STRUCTURE

INTERACTIONS WITH TUBULIN

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Abstract—Cornigerine is a natural product analog of colchicine produced by *Colchicum cornigerum* in which the vicinal 2- and 3-methoxy groups are condensed into a methylenedioxy bridge. This produces a fourth ring and a structure which resembles a hybrid of colchicine, podophyllotoxin, and steganacin. Cornigerine was somewhat more toxic than colchicine with L1210 murine leukemia cells and caused them to accumulate in metaphase arrest. Cornigerine resembled colchicine in its interactions with tubulin *in vitro*, and it was also somewhat more potent than colchicine in these drug–tubulin interactions. Cornigerine inhibited tubulin polymerization both with and without microtubule-associated proteins, inhibited the binding of radiolabeled colchicine to tubulin, and stimulated tubulin-dependent GTP hydrolysis. Indirect evidence suggested that the binding of cornigerine to tubulin is relatively slow and temperature-dependent, like the binding of colchicine to the protein.

The antimitotic drug colchicine has long been of interest in biology and medicine. A major advance in understanding its mode of action was made almost 20 years ago when it was used as a key element in the initial purification of tubulin, the major protein constituent of microtubules [1]. The drug–protein complex, even though no covalent bond was formed, remained largely intact throughout its purification. Consequently, colchicine was established as an inhibitor of tubulin polymerization, for microtubules form the framework of the mitotic spindle absent in colchicine-treated cells. A great deal of interest has subsequently been focused on structural features of colchicine responsible for its interactions with tubulin, and many drugs compete with colchicine in binding to the protein [2–11]. In some cases, structural analogy to colchicine is obvious [2–9], whereas in others it is quite obscure [9–11].

Our laboratory is currently studying the interactions with tubulin of a new series of natural products derived from the bark of the South African tree *Combretum caffrum* [4, 5]. Isolated by Pettit and his collaborators [5, 12], the first compounds obtained were biphenyls, including a trimethoxybenzene ring, with the two benzene rings linked by a two-carbon bridge. Their structural analogy to colchicine was obvious [4, 5]. More recently, however, a potent analog was isolated in which the trimethoxybenzene ring was replaced by a benzodioxole ring system (presumably by an enzymatic condensation of two vicinal methoxy groups), resulting in greater structural analogy to podophyllotoxin than to colchicine (manuscript in preparation).

As a consequence of this observation, we surveyed the literature more thoroughly and became aware of an alkaloid extracted from *Colchicum cornigerum* known as cornigerine [13, 14]. This compound, whose structure is presented in Fig. 1, would appear to be derived directly from colchicine by an enzymatic route analogous to that employed by *Combretum caffrum*. While cornigerine has been included in surveys of colchicine analogs [15, 16], which indicated that it had strong antitubulin activity, its biological properties have not been examined thoroughly. Its unique structure, a blend of features associated with colchicine, podophyllotoxin and steganacin (Fig. 1), led us to evaluate its antimitotic properties and its interactions with tubulin in detail.

MATERIALS AND METHODS

Materials. Electrophoretically homogeneous bovine brain tubulin and heat-treated microtubule-associated proteins were prepared as described previously [17]. GTP and [8-^{14}C]GTP were repurified by ion-exchange chromatography on DEAE-Sephadex A-25 by triethylammonium bicarbonate gradient elution. Colchicine was from Sigma (St. Louis, MO), [^{3}H]colchicine from Amersham (Arlington Heights, IL), and podophyllotoxin from Aldrich (Milwaukee, WI). Cornigerine and steganacin were gifts, respectively, of Dr. V. Simanek and Dr. J-P. Robin. All nonradiolabeled drugs were dissolved in dimethyl sulfoxide. The solvent was present in control reaction mixtures in amounts equivalent to those added with the drugs, and this had no effect on the reactions being examined.

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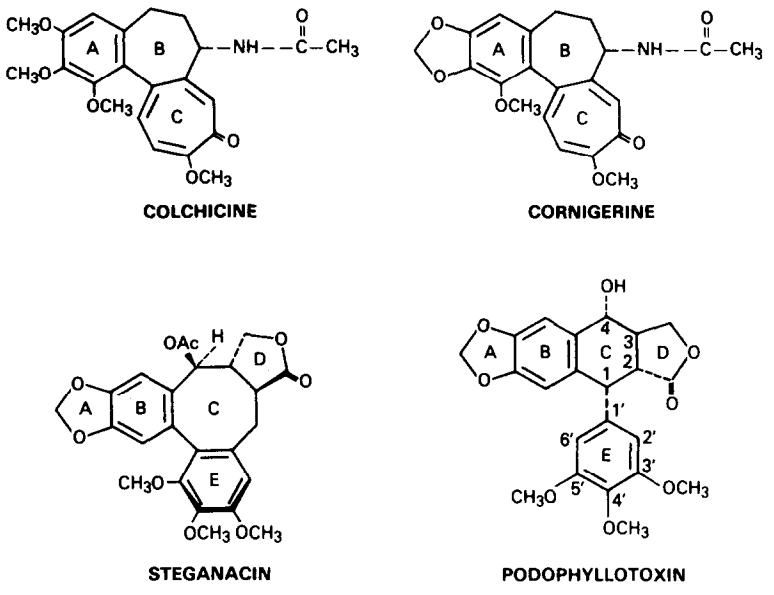

Fig. 1. Structures of colchicine, cornigerine, steganacin, and podophyllotoxin.

Methods. Tubulin polymerization was followed turbidimetrically, and the binding of radiolabeled colchicine to tubulin was measured by the DEAE-cellulose filter method as described previously [7]. GTP hydrolysis was measured by following the formation of [8-^{14}C]GDP, using thin-layer chromatography on polyethyleneimine-cellulose and autoradiography [18]. Culture of L1210 murine leukemia cells and evaluation of cultured cells for mitotic arrest were performed as described previously [4].

RESULTS AND DISCUSSION

Our initial study with cornigerine was an evaluation of its toxic effect on the growth of L1210 murine leukemia cells, together with a demonstration that it caused the accumulation of cells arrested in mitosis at cytotoxic drug concentrations (Fig. 2). The IC_{50} values obtained for cornigerine ranged from 0.02 to 0.04 μM, whereas those for colchicine varied from 0.06 to 0.1 μM. For comparison, the averages of values obtained for podophyllotoxin and steganacin were 0.01 and 0.06 μM respectively. Thus, cornigerine has cytotoxic activity comparable to other colchicine analogs and is somewhat more inhibitory than colchicine itself.

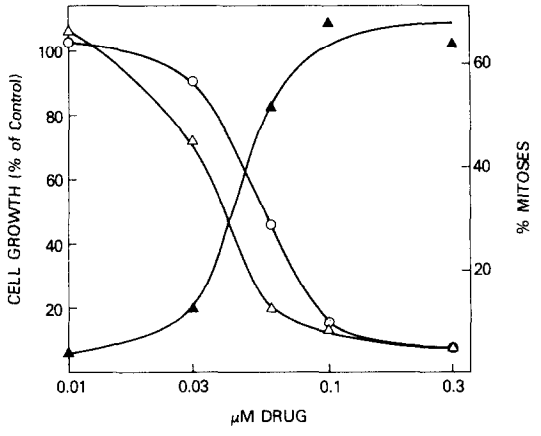

Fig. 2. Effects of cornigerine on growth and mitosis of L1210 murine leukemia cells. Cultures were incubated for 24 hr with the indicated concentrations of colchicine (○) or cornigerine (△). Cell number in control cultures increased from 2.1×10^5 to 8.7×10^5 after 24 hr. Cell cultures were also evaluated for mitotic arrest with the indicated concentrations of cornigerine (▲) after 6 hr of growth.

We next confirmed [15] that cornigerine was effective in inhibiting the binding of radiolabeled colchicine to tubulin (Table 1). This assay was performed under conditions in which the radiolabeled colchicine was subsaturating and with a short incubation time, while the amount of drug bound to protein was rising rapidly. Hence, equimolar nonradiolabeled colchicine did not inhibit binding of the radiolabeled drug by 50% as would occur with

Table 1. Inhibition of the binding of [^{3}H]colchicine to tubulin by cornigerine

Nonradiolabeled drug added	% of Control (range; standard deviation)
Colchicine	77 (71–80; 4.5)
Cornigerine	54 (49–57; 4.0)
Podophyllotoxin	13 (11–13; 1.2)
Steganacin	49 (44–49; 2.9)

Each reaction mixture contained 1 μM tubulin, 5 μM [^{3}H]colchicine, and the indicated nonradiolabeled drug at 5 μM. Incubation was for 10 min at 37°. Average values obtained in three independent experiments, together with the range of values and the standard deviation from the mean, are presented. With the control reaction mixtures, an average of 30.5 pmol (range 26.8 to 33.1 pmol) of [^{3}H]colchicine was retained by the DEAE-cellulose filters.

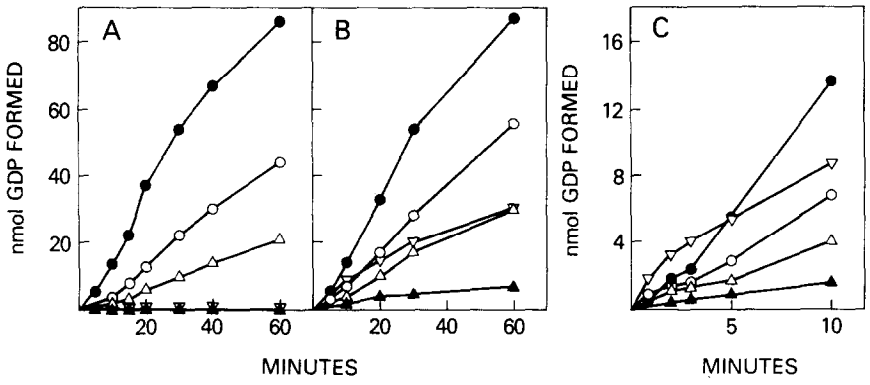

Fig. 3. Effects of cornigerine, colchicine, steganacin, and podophyllotoxin on tubulin-dependent GTP hydrolysis. (A) GTP hydrolysis without microtubule-associated proteins. (B) GTP hydrolysis with heat-treated microtubule-associated proteins. (C) Early time points in the reactions with microtubule-associated proteins. Each 100-μl reaction mixture contained 0.1 M 4-morpholineethanesulfonate (pH 6.4 with NaOH), 0.5 mM $MgCl_2$, 10 μM tubulin (1.0 mg/ml), 0.1 mM [8-^{14}C]GTP, 0.5 mg/ml microtubule-associated proteins as indicated above, and 0.1 mM drug, as indicated by the following symbols: (∇) none; ($\circ$) colchicine; ($\bullet$) cornigerine; ($\triangle$) steganacin; and ($\blacktriangle$) podophyllotoxin. Reaction mixtures were incubated at 37°, and at the indicated times 10-μl aliquots were removed from the reaction mixtures, processed, and analyzed. Data are expressed as nmol GDP formed/ml reaction.

saturating drug at equilibrium. In this assay, too, cornigerine was somewhat more effective than colchicine, but it was slightly less active than steganacin and much less inhibitory than podophyllotoxin.

In agreement with the results of Dvorackova *et al.* [16], we found that cornigerine was also more potent than colchicine as an inhibitor of microtubule assembly (data not presented, but see below). We compared its effect to that of colchicine in a reconstituted system containing tubulin plus heat-treated microtubule-associated proteins [17].*

The effect of cornigerine on tubulin-dependent GTP hydrolysis was particularly noteworthy (Fig. 3). This reaction has been either stimulated or inhibited by every mitotic agent yet examined [4, 7, 8, 19, 20], and cornigerine was no exception to this generalization. Figure 3 presents the time course of the reaction at low ionic strength both in the absence (Fig. 3A) and presence (Fig. 3B and C) of heat-treated microtubule-associated proteins. The reactions with no drug were compared to those with cornigerine, colchicine, podophyllotoxin, and steganacin. It should be emphasized that, at the drug concentrations used in the experiments of Fig. 3, microtubule assembly was inhibited totally by all four antimitotic agents.

Without microtubule-associated proteins there was minimal hydrolysis without drug, and podophyllotoxin further suppressed this reaction (not apparent in Fig. 3A, but see Ref. 7). The three other compounds all stimulated GTP hydrolysis to various extents, and the most extensive stimulation occurred with cornigerine.

With microtubule-associated proteins, significant GTP hydrolysis, initially simultaneous in onset and stoichiometric with microtubule assembly [21], occurred in the absence of drugs (Fig. 3B and C) (the heat-treated microtubule-associated proteins alone have no GTPase activity; see Ref. 17). Podophyllotoxin, as demonstrated previously [7, 19, 20], inhibited this reaction at all time points. The early phase of hydrolysis, most closely associated with the assembly reaction [21], was inhibited by the other three drugs as well (Fig. 3C), but at later time points cornigerine, colchicine, and steganacin all stimulated net GTP hydrolysis (Fig. 3B). As in the absence of microtubule-associated proteins, the most vigorous stimulation occurred with cornigerine and the least vigorous with steganacin. With colchicine and steganacin, as well as with podophyllotoxin (but not cornigerine), GTP hydrolysis was somewhat greater in the presence of microtubule-associated proteins than in their absence. Since there was no concurrent assembly reaction, the reason for this is unclear.

A major characteristic of the binding of colchicine to tubulin is that the reaction is slow and does not occur at 0°. At the present time cornigerine is not available in a radiolabeled form and, therefore, its binding to tubulin cannot be examined directly. Tubulin-dependent GTP hydrolysis in the presence of low concentrations of drugs is a way to approach this question indirectly, for colchicine stimulation of GTP hydrolysis at lower drug concentrations is enhanced markedly by a drug–tubulin preincubation at 37° prior to addition of radiolabeled GTP [4, 20]. Table 2 demonstrates that stimulation of hydrolysis by both cornigerine and colchicine at 2 and 3 μM displayed this behavior, although some stimulation

* In a series of experiments with reaction mixtures containing 1.0 mg/ml tubulin, 0.5 mg/ml heat-treated microtubule-associated proteins, 0.1 M 4-morpholineethanesulfonate (pH 6.4 with NaOH), 0.5 mM $MgCl_2$ and 0.4 mM GTP, microtubule assembly was inhibited by 50% with 8–9 μM colchicine or with 4–5 μM cornigerine. If drug and proteins were preincubated prior to assembly, both compounds were significantly more potent. Although this effect was not quantitated carefully, cornigerine remained more potent than colchicine following preincubation. In an experiment with the drugs at 3 μM, assembly was inhibited 52% by colchicine and 80% by cornigerine.

Table 2. Enhancement of stimulation of tubulin-dependent GTP hydrolysis by colchicine and cornigerine with a drug–protein preincubation

Drug	% of Control (range; standard deviation)	
	Nonpreincubated	Preincubated
Colchicine, 2 μM	104 (103–105; 1.2)	144 (142–146; 2.0)
Colchicine, 3 μM	106 (104–107; 1.5)	184 (177–192; 7.6)
Cornigerine, 2 μM	118 (113–121; 4.2)	173 (162–182; 10.1)
Cornigerine, 3 μM	140 (134–145; 5.5)	254 (244–262; 9.2)

Each 50-μl reaction mixture contained 10 μM tubulin, 1 M monosodium glutamate (pH 6.6 with HCl), 0.1 mM [8-^{14}C]GTP, 1.0 mM $MgCl_2$, and drugs as indicated. Incubation was for 20 min at 37°. In preincubated samples all components except GTP were mixed in a 45-μl volume, warmed to 37° for 10 min, and chilled on ice; [8-^{14}C]GTP was added; and the 37° incubation was resumed. Average values obtained in three independent experiments, together with the range of values and the standard deviation from the mean, are presented. With the nonpreincubated control reaction mixtures, an average of 15.3 nmol/ml of [8-^{14}C]GDP was formed (range 13.5 to 16.7 nmol/ml); with the preincubated control reaction mixtures, an average of 14.2 nmol/ml of [8-^{14}C]GDP was formed (range 12.8 to 15.0 nmol/ml).

was observed with cornigerine (but not colchicine) in the nonpreincubated samples.

Similar enhancement of the effects of low concentrations of both colchicine and cornigerine was demonstrated in their inhibitory effects on tubulin polymerization. Figure 4A presents an experiment in which 2 μM colchicine and cornigerine were compared without a drug–tubulin preincubation. Although some inhibition was observed with cornigerine, the reaction with colchicine differed little from that in the drug-free control. Preincubating drug with tubulin prior to the addition of GTP clearly enhanced the inhibitory effects of both drugs (Fig. 4B), and cornigerine remained more inhibitory than colchicine (with both present at the same concentration). Similar relative activity of both agents was

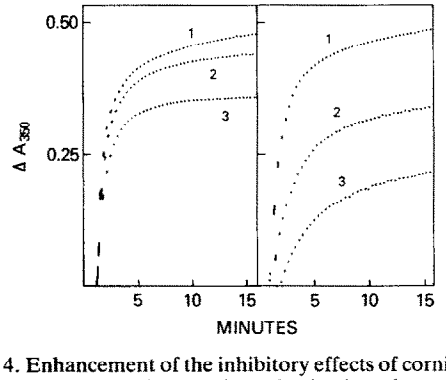

Fig. 4. Enhancement of the inhibitory effects of cornigerine and colchicine on tubulin polymerization by a drug–protein preincubation. Each 0.25-ml reaction mixture contained 10 μM tubulin (1.0 mg/ml), 1 M monosodium glutamate (pH 6.6 with HCl), 1.0 mM $MgCl_2$, and either no drug (curves 1), 2 μM colchicine (curves 2), or 2 μM cornigerine (curves 3). In the experiments presented in panel A, 1 mM GTP was included in the reaction mixtures, and polymerization was examined turbidimetrically at 37° without a protein–drug preincubation. In the experiments presented in panel B, the reaction mixtures were incubated for 10 min at 37° and chilled on ice, GTP was added to 0.4 mM, and then polymerization was examined turbidimetrically at 37°.

obtained in a polymerization system dependent on heat-treated microtubule-associated proteins (data not presented; cf. Ref. 16).

To summarize these experimental findings, cornigerine is a highly active natural product analog of colchicine in which the methoxy groups at positions 2 and 3 have been condensed into a methylenedioxy bridge. The biological properties of cornigerine seemed to differ little from those of colchicine, except that cornigerine was somewhat more active than colchicine in all systems examined, including its toxicity for L1210 murine leukemia cells.

The structures of colchicine, cornigerine, steganacin, and podophyllotoxin (Fig. 1) almost form a continuum. These agents have obvious structural analogies with each other, but there is no feature common to all four compounds. The methylenedioxybenzene ring system present in cornigerine, podophyllotoxin and steganacin is absent in colchicine; the trimethoxybenzene ring of steganacin, colchicine and podophyllotoxin is missing in cornigerine; and only colchicine and cornigerine have a tropolone ring. Thus, no single structural feature can be essential for the strong tubulin–drug interaction at the colchicine binding site which occurs with these four natural products.

A number of workers have attempted to ascribe specific functions to the different component parts of molecules binding at the colchicine site, particularly when structural analogies to colchicine and podophyllotoxin are obvious [2–4, 6, 7, 9, 20, 22–25]. It seems likely, however, in view of the great variety of molecules which bind at this site [2–11], that specific structural features are of much less importance than the overall conformation of a potential ligand. The colchicine site is probably a complex hydrophobic pocket on tubulin, requiring multiple points of contact with small molecules which bind in it with high affinity. The great variety of compounds which bind in this site suggests that these contacts can be made in many ways, which are nonetheless highly specific. The relatively slow binding of colchicine and, probably, cornigerine may require, in addition, conformational changes in the drug, the

protein, or the drug–protein complex [26]. It is clear, however, that conclusions about structure–activity relationships must be made with extreme caution.

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