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N-Alkyl colchiceineamides: their inhibition of GTP or taxol-induced assembly of tubulin

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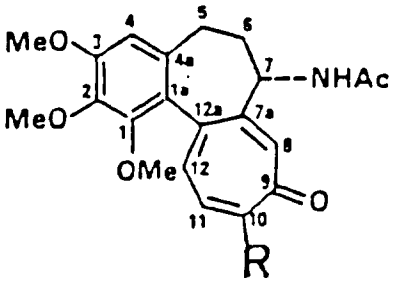
Colchicine is a potent drug that interferes with microtubule assembly both *in vitro* and *in vivo*, by forming a complex with the dimeric subunit of the microtubule, tubulin [1]. The complex formed incorporates into the microtubule and inhibits the assembly process [2, 3].

Many colchicine derivatives have been extracted from plants or synthesized over the years [4]. We have synthesized a group of 10-*N*-alkyl derivatives of colchicine (see Table 1) in order to examine their anti-microtubule activities. The 10-methoxy position of colchicine is known to be liable to mild hydrolysis [4]. In contrast, the 10-amino derivatives are resistant to hydrolysis due to the high pK_a values of the amine. The activities of these compounds as tubulin assembly inhibitors were studied using GTP and taxol as promoters of tubulin assembly, and in cells. GTP is an essential component in the tubulin assembly mechanism and will promote assembly of uninhibited protein under proper experimental conditions [5]. Taxol, a novel diterpenoid, promotes the assembly of microtubules under conditions that normally do not support microtubule polymerization, by interacting directly with microtubule polymers and stabilizing them against depolymerizing agents such as Ca^{2+} and cold temperature [6]. Using both agents, we have studied the structure–activity relationships of colchiceineamides.

Materials and methods

Preparation of colchicine analogs. To 1-ml solutions of colchicine (200 mg, 0.5 mmol) in dry acetonitrile, several amines (20 mmol) were added: methyl-, ethyl-, propyl-, isopropyl-, butyl-, isobutyl-, *tert*-butyl-, 3-dimethyl-amino-1-propyl-amine, 1,6-hexadiamine and dimethylhydrazine (Table 1 summarizes the analogs prepared). The reactions were carried out at room temperature (except for the reaction of 1,6-hexadiamine which was carried out in a hot water bath). The formation of the desired products was monitored by TLC. Retention times (R_f) for the 10-amino derivatives that eluted on Alumina (5:95 ethanol–CH_2Cl_2) were: 55, 55, 53, 54, 57, 45, 50, 56, 35 and 62% respectively. Silica TLC plates (1 mm) were used for preparative separation. The reaction mixtures were eluted by ethanol–CH_2Cl_2 (2.5:97.5), and the appropriate compounds were extracted from the silica by hot chloroform and recrystallized from an ethylacetate–hexane mixture. The melting points of the products were as follows: 150–155, 160–165, 110, 150, 110–115, 220, 155–160, 205, 160, and 110–120° respectively. The structures of the resulting compounds were confirmed by NMR measurements (Varian 400 MHz spectrometer), mass spectrometry, elemental analysis and, in one case (compound IX), by single crystal X-ray

Table 1. 10-*N*-Alkyl derivatives of colchicine, their structures and their potencies as inhibitors of tubulin assembly *in vitro*



		R	% Assembly* With GTP	% Assembly* With taxol
I	Colchicine	OCH_3	17	48
II	*N*-methyl colchiceineamide	$NHCH_3$	<5	8
III	*N*-Ethyl	$NHCH_2CH_3$	<5	15
IV	*N*-Propyl	$NHCH_2CH_2CH_3$	30	57
V	*N*-Isopropyl	$NHCH(CH_3)_2$	33	62
VI	*N*-Butyl	$NHCH_2CH_2CH_2CH_3$	90	92
VII	*N*-Isobutyl	$NHCH_2CH(CH_3)_2$	94	95
VIII	*N*-*tert*-Butyl	$NHC(CH_3)_3$	96	95
IX	*N*-3-Dimethylamino-1-propyl	$NHCH_2CH_2CH_2N(CH_3)_2$	98	100
X	*N*-6-Hexylamine	$NH(CH_2)_6NH_2$	104	103
XI	*N*-$N(CH_3)_2$	$NHN(CH_3)_2$	23	35

* Percent assembly is the ratio between the steady-state value of the assembly of tubulin in the presence of drug (5 μM) to that without drug. The samples (1.2 mg/ml) were incubated for 30 min at 37°, prior to the addition of 1 mM GTP or 10 μM taxol.

crystallography. Solutions of each drug, for tubulin assembly studies or LC_{50} determinations, were prepared in dimethyl sulfoxide (DMSO). The final concentrations of DMSO in tubulin assembly studies were less than 1% and for LC_{50} determinations, 0.1% v/v.

Tubulin preparation and assembly measurements. Tubulin was prepared from calf brains by three cycles of polymerization–depolymerization according to a procedure modified from Shelanski *et al.* [5]. Tubulin stocks were stored at −20° in MES buffer consisting of 0.1 M 2(*N*-morpholino) ethanesulfonic acid (MES), 2 mM ethylene bis (oxyethylenenitrilo) tetraacetate (EGTA), 0.5 mM $MgCl_2$ and 5 M glycerol, pH 6.7. Stock aliquots were diluted 1:1 with glycerol-free MES buffer, and the working sample was clarified by centrifugation at 30,000 rpm for 15 min at 4° prior to use.

Tubulin samples were incubated at 37° with or without drug for 30 min prior to the addition of GTP (1 mM) or taxol (10 μM). This time period allows for the formation of drug–tubulin complex which, at least for colchicine, is essential for the inhibition of tubulin assembly [7]. The change in microtubule mass concentration was monitored spectrophotometrically as a change in the absorbance at 350 nm with time [8]. Samples were examined by electron microscopy.

Competition between colchicine and its analogs for the tubulin binding site. Samples of tubulin were incubated for 45 min at 37° with a mixture of a fixed concentration of [^{3}H]colchicine (New England Nuclear) and different concentrations of its analogs. The colchicine binding assay was performed according to Borisy [9]. Briefly, 100-ml aliquots of the incubated mixture were applied to a stack of three DEAE-cellulose paper discs (Whatman, DE81, 2.5 cm in diameter) and washed three times with MES buffer. The discs were placed in a counting bottle containing scintillation fluid (Lumax–toluene, 1:3), and samples were counted after 1 day.

LC_{50} *of cells in the presence of colchicine and its analogs.* The synthesized compounds were tested, as cell growth inhibitors, on several cell lines. In all of these experiments, the cells were incubated with or without drug for 72 hr (before the control samples reached confluency), and the number of living cells was determined, using a Coulter counter. For each drug, at least three dishes were prepared for each concentration. The appropriate procedures for

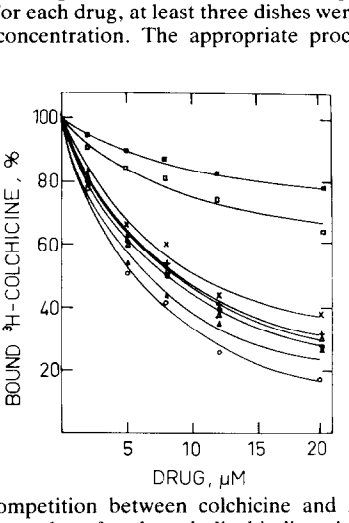


Fig. 1. Competition between colchicine and its *N*-alkyl colchiceine analogs for the tubulin binding site. Tubulin (1.0 mg/ml) was premixed with 8 μM [^{3}H]colchicine at 4°. Various concentrations of drug were added to 250-μl aliquots of the mixture. The samples were processed as described under Materials and Methods. The drugs presented in this figure are: cold colchicine (I, ●), *N*-Me (II, ○), *N*-Et (III, ▲), *N*-Pr (IV, △), *N*-isoPr (V, ×), *N*-Bu (VI, □), *N*-isoBu (VII, ■) and *N*-$N(CH_3)_2$ (XI, +) colchiceineamides.

Table 2. Drug sensitivity of several cell lines to colchiceineamides

Cell line	LC_{50}^{*} (μM)								
	I	II	III	IV	V	VI	VII	IX	XI
P388	0.029	0.029	0.029	0.036	0.048	0.24	0.32	>10	0.025
P388/ADR	0.32	0.3	0.32	0.54	0.32	3.2	3.6	>10	0.34
J774.2	0.035	0.035	0.044	0.158	0.053	1.0	1.0	>10	0.035

LC_{50}, drug concentration that inhibits cell division by 50% after 72 hr.

treating each cell line are described in the following publications: J774.2 [10] and P388 and P388/ADR [11].

Results and discussion

The analogs studied are outlined in Table 1. As can be seen, for tubulin samples that were incubated with equiconcentrations of drugs for the same length of time, it is clear that substitution of the 10-methoxy group did not reduce the activity of the drug as long as the alkyl moiety was short. In fact, methyl (II) and ethyl (III) amines were even more potent than colchicine. Propyl (IV) and isopropyl (V) were comparable to colchicine, while larger alkyl groups, from butyl (VI, VII and VIII) derivatives and larger, had almost no inhibitory potency on tubulin polymerization. The dimethylhydrazine (XI) derivative was as potent as colchicine. With higher concentrations of these analogs or longer incubation times prior to GTP or taxol introduction, less polymer was assembled.

Studies on the competition of each colchicine derivative and [^{3}H]colchicine are summarized in Fig. 1. The results clearly indicate that all the analogs tested competed with colchicine for the same binding site on tubulin and their relative affinities to this site dictated their poisoning potencies on tubulin assembly, as seen in Table 1.

Several drug-treated cell lines showed a decrease in their viability and proliferation, and this was compatible with their effect on tubulin assembly. Table 2 summarizes the LC_{50} for three cell lines. The LC_{50} for the doxorubicine-resistant line (P388/ADR) was about one order of magnitude higher than the LC_{50} for the native P388 line, due to cross-resistancy to colchicine and its analogs. An immunofluorescence study with antibody directed against tubulin performed on CHO cells treated with several of these drugs (not shown) revealed the disappearance of the microtubule cytoskeleton. These results demonstrate that the colchicine analogs appear to function on cell lines in a manner similar to the parent compound.

To summarize, we have presented a series of colchicine derivatives which guard the C-10 position from being hydrolyzed. The potencies of these drugs as tubulin assembly inhibitors were reciprocal to the size of the amino substituent. These drugs can also serve as precursors for further modification of the colchicine molecule.

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In vivo binding of [1-^{14}C]methylisocyanate to various tissue proteins

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Cyanates have been reported to be antisickling agents which bind to the N-terminal valine of α- and β-chains of haemoglobin [1, 2]. *In vivo* carbamylation of various tissue proteins, including brain proteins by cyanate, has been reported [3, 4]. Isocyanates, the reactive isomers of cyanates, also bind with proteins [2], and methylisocyanate (MIC) has been shown to be an effective antisickling agent *in vitro* [5]. Use of cyanate as an antisickling agent *in vivo* is limited due to its high toxicity [6]. There is a paucity of literature on MIC binding to various tissue proteins after