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Synthesis and Binding to Tubulin of an Allocolchicine Spin Probe

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A spin probe for the colchicine binding site on tubulin has been synthesized from allocolchicine. The probe is competitive to colchicine with an apparent inhibition constant of 11 μM while allocolchicine has an inhibition constant of 2 μM. Microtubule assembly is inhibited to 50 % by 7 μM of the spin probe. As a mitotic poison the spin probe is less potent than colchicine. These data suggest that the probe binds to the same site on tubulin as colchicine and in spite of the somewhat lower efficiency, it seems to be a valuable tool for the study of the microtubule system.

Colchicine (*1*) is one of the antimitotic drugs that interfere with the function of microtubules.[1] The tubulin dimer, *i.e.*, the subunit protein of microtubules, has one binding site for colchicine.[2,3] The binding of colchicine to tubulin is a rather slow process which exhibits a high temperature coefficient.[4,5] The binding is non-covalent and almost irreversible. The complex between tubulin and colchicine can be isolated.[6,7] The unique property to bind colchicine has become a major criterion for the identification of tubulin.

It has been suggested that the binding of colchicine produces a conformation change in tubulin,[8,9] or in colchicine.[10] It is possible that the tubulin-colchicine complex is incorporated into microtubules at their assembly end and forms a "cap", blocking further addition of tubulin.[11] Alternatively, the complex might copolymerize and reduce the affinity of the assembly end for tubulin.[5,12] Further, colchicine has been reported to bind to the 36 S double rings, the tubulin oligomers present in depolymerized microtubule protein,[13] and the drug might also be involved in the process of nucleation.[14]

To obtain further information about the influence of colchicine on the microtubule assembly, and about the mechanism of its binding to tubulin, we have studied these interactions by the technique of spin labelling. A prerequisite for such studies was the synthesis of a colchicine spin probe which exhibits the characteristic properties of colchicine, *i.e.*, inhibition of microtubule assembly, high affinity binding to tubulin and antimitotic activity in cell cultures. In addition, the spin probe has to be a competitor to colchicine for the same site on tubulin.

RESULTS AND DISCUSSION

Allocolchicine (*2*) has been found to be a potent inhibitor for the microtubule system.[15] We have found that allocolchicine competes with colchicine for the same site on tubulin (Fig. 1) with an apparent inhibition constant of 2 μM.

By converting the troponoid C-ring of colchicine to the benzoid ring of allocolchicine (*2*), the problems associated with photoisomerization and positioned isomerism are avoided. Desacetylallocolchicine (*3*), previously reported,[19] but not characterized, has been prepared by us in two steps from colchicine in high yield. Acylation of *3* with the acid *4* (4-glutaramido-2,2,6,6-tetramethyl-1-piperidinyloxy) gave the spin probe (*5*). The spin probe (*5*) was strongly bound to tubulin, and the complex could be isolated by gel chromatography on Sephadex G25 at 4 °C. The complex was rather stable at 4 °C, but the spin probe was slowly released at room temperature as observed by EPR spectroscopy. The binding of the

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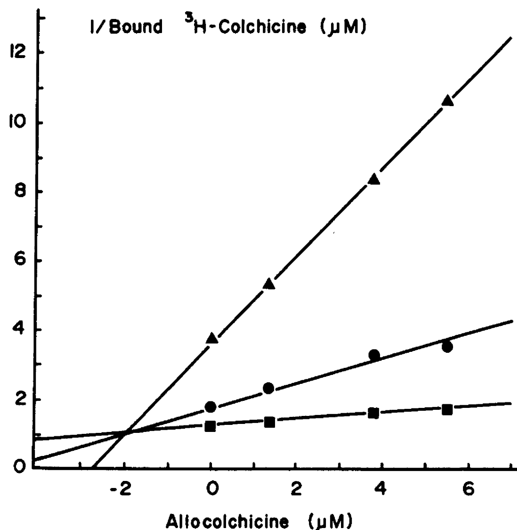


Fig. 1. Modified Dixon Plot[20] indicating a competition between allocolchicine and colchicine for binding to the same site on tubulin. Tubulin (1.94 µM) was incubated with ^{3}H-colchicine: 8.2 (■), 2.66 (●), and 0.899 µM (▲), and varying concentrations of allocolchicine in assembly buffer at 37 °C for 90 min. The reaction was quenched at 0 °C, and tubulin was subsequently isolated on DEAE cellulosa filter paper.[21] Radioactivity was then measured after extraction overnight.

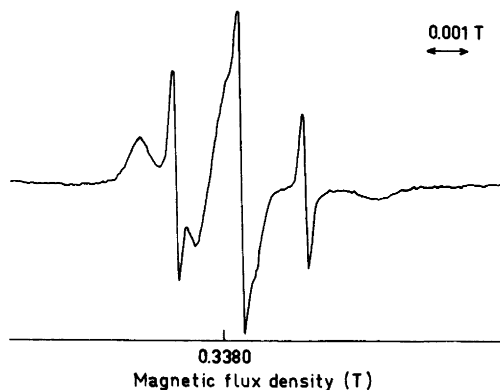
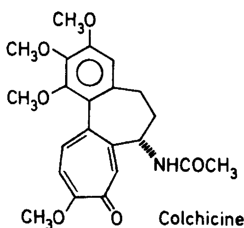
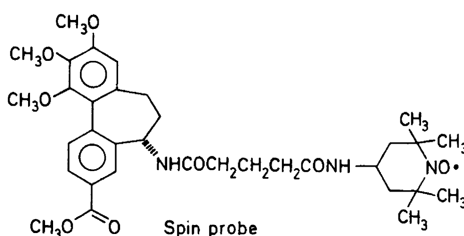
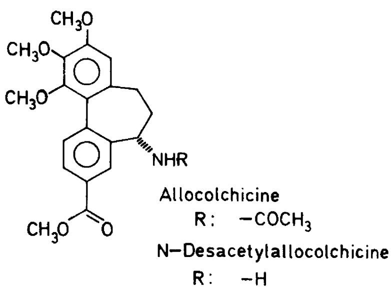


Fig. 2. EPR spectrum of the spin probe (5) in the presence of tubulin. Tubulin (100 µM) was incubated with an equimolar amount of the spin probe in assembly buffer at 37 °C for 1 h, and separated from unbound spin probe by chromatography on Sephadex G25 at 4 °C. The remaining amount of unbound spin probe (the sharp lines in the spectrum) constituted only a small percentage of the total amount of the probe. The EPR spectrum was recorded at 20 °C, a frequency of 9 GHz, a microwave power of 5 mW, and with a modulation amplitude of 0.1 mT.







spin probe is non-covalent as the probe was completely detached after the addition of guanidine-HCl to a concentration of 4 M. EPR spectroscopy showed that the spin probe was highly immobilized by the binding to tubulin (Fig. 2). The line-width of the low field line was about 30 % broader than the corresponding line in the spectrum obtained from tubulin spin labelled with 3-maleimido-2,2,5,5-tetramethylpyrrolidinyloxy, at the two most reactive sulfhydryl groups of tubulin.[16] The rela-

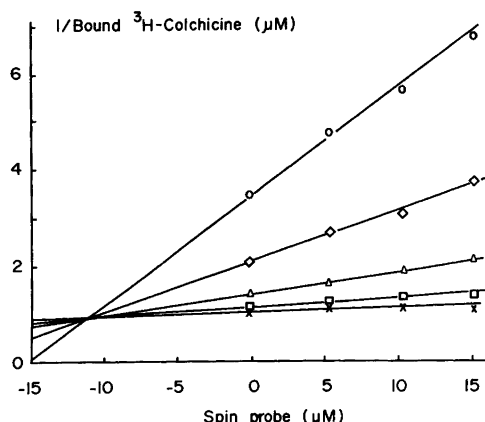


Fig. 3. Modified Dixon plot indicating a competition between the spin probe (5) and colchicine for binding to the same site on tubulin. Tubulin (1.92 μM) was incubated with ^{3}H-colchicine: 13.5 (×), 6.6 (□), 3.16 (△), 1.55 (□), and 0.775 μM (○) and varying concentrations of the spin probe in assembly buffer as described in Fig. 1.

tively broad line seems to indicate that the spin probe is firmly attached to tubulin, perhaps by location in a cleft in the protein molecule, or by a high degree of hydrophobic interaction. Assembly of microtubule proteins (2.5 mg/ml) into microtubules was inhibited to 50 % by 7 μM of the spin probe. The binding of ^{3}H-colchicine at 37 °C to purified tubulin was competitively inhibited by the spin probe (Fig. 3). An apparent inhibition constant of 11 μM was evaluated from the inhibition experiment. The competitive inhibition suggests that the spin probe (5) and colchicine are bound to the same site on tubulin. However, both the efficiency to inhibit assembly and the binding strength indicate that the spin probe is less effective than colchicine as the inhibition constants differ about one order of magnitude from the corresponding values observed with colchicine.[1,15] As a mitotic poison the spin probe (5) is less potent than colchicine. In a culture of neuroblastoma cells, cell proliferation was inhibited to 50 % by the the spin probe in concentrations in the order of one μM (Table 1).

In spite of the somewhat lower efficiency than colchicine the spin probe (5) seems to be a useful tool for the study of the active site of assembly, and to constitute a reporter group for the size of the aggregates involved in the inhibition process.

Table 1. Effects of colchicine derivatives on cell proliferation.

	50 % inhibition of cell proliferation (nM)
Colchicine	12
Allocolchicine	15
Spin probe (5)	1200

EXPERIMENTAL

Melting points were determined on a Kofler hot bench and specific rotations on a Perkin Elmer 141 polarimeter. UV spectra were recorded on a Perkin Elmer 571 spectrophotometer, mass spectra on an AEI MS 902, NMR spectra on a Bruker WH 270 and EPR spectra on a Varian E-9 spectrometer. TLC data are given in Table 2.

Synthesis of the spin probe (5). Allocolchicine was prepared by base catalyzed rearrangement of colchicine.[17,18]

4-Glutaramido-2,2,6,6-tetramethyl-1-piperidinyloxy (4). Glutaric anhydride (1.2 g, 11 mmol) in 10 ml of acetone was added to 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy (1.7 g, 10 mmol) in 10 ml of the same solvent. The reaction mixture was kept in a refrigerator overnight. The crystalline precipitate was collected and recrystallized from acetone to give 1.5 g of pure *4*, red prisms (acetone – toluene) m.p. 139 – 140 °C.

Table 2. Thin layer chromatography on Merck Kieselgel 60 F 254. Spots were located with UV light, by exposition to iodine vapor, or by treatment of the chromatogram with concentrated nitric acid. With the latter reagent allocolchicine derivatives give rise to violet and colchicine derivatives to yellow spots.

The solvent systems used were: (A) Chloroform – acetone – 2-propanol 10:5:1 (v/v), (B) chloroform – diethyl ether – acetic acid 10:8:2 (v/v), (C) chloroform – cyclohexane – diethylamine 10:8:2 (v/v).

Compound No.	R_F values Solvent system A	B	C
1	0.13	0.05	0.11
2	0.45	0.32	0.24
3	0.33	0.05	0.41
4	0.18	0.20	0
5	0.35	0.16	0.17

Desacetylallocolchicine (3). Allocolchicine (1.5 g) in 50 ml of methanol containing 4 % sulfuric acid was heated in a stoppered tube at 65 °C until no starting material could be detected by TLC. The reaction usually required 10 – 14 days. The cooled reaction mixture was then poured on an equal volume of ice, and was subsequently made strongly basic by the addition of potassium hydroxide solution. The mixture was extracted with chloroform, the extract was washed with water, and evaporated to dryness to give *3* as the amorphous base in a chromatographically pure state, yield 1.2 g, $[\alpha]_D^{22}$ – 147° (*c* 0.3, ethanol) MS: [m/e (% rel. int.)] 357 (78,M), 340 (100, [M – NH_3]) Mol.wt., obs.: 357.1572 calc. for $C_{20}H_{23}NO_5$: 357.1576 ^{1}H NMR (270 MHz, $CDCl_3$): δ 1.74 (1H,m), 2.22 (1H,m), 2.43 (2H,m), 3.61 (3H,s), 3.84 (1H,dd), 3.89 (6H,s), 3.93 (3H,s), 6.58 (1H,s), 7.50 (1H,d), 7.97 (1H,d), 8.28 (1H,s), amino protons were not detected.

Acetylation of *3* with acetic acid and *N,N'*-di-(cyclohexyl)carbodiimide gave a quantitative yield of allocolchicine, identical with the starting material (TLC, optical rotation, m.p. and mixed m.p.).

*"Spin probe", 5 4-[{5-[(6,7-dihydro-3-methoxy-carbonyl-9,10,11-trimethoxy-5*H*-dibenzo*[a,c]*cyclo-hepten-(5*S*)-yl)amino]-1,5-dioxopentyl}amino]-2,2,6,6-tetramethyl-1-piperidinyloxy.* To a stirred solution of *3* (0.22 g, 0.6 mmol) in 6 ml of dichloromethane, powdered *4* (0.26 g, 0.9 mmol) and an *N,N'*-di(cyclohexyl)carbodiimide solution (3 ml, 0.3 M in tetrachloromethane) were added each in small portions. The addition of the reagents required 90 min, after which time TLC showed only traces of *3*. After an additional 30 min at room temperature the reaction mixture was filtered to remove precipitated *N,N'*-di(cyclohexyl)urea. Evaporation of the filtrate to dryness under reduced pressure and repeated crystallization from benzene gave 0.25 g of pure *5*, pink needles, m.p. 217 – 218 °C. $[\alpha]_D^{22}$ –95° (*c* 0.1, ethanol). UV (96 % ethanol) λ max 291 nm (log ε 4.20), MS: [m/e (% rel. int.)] 624 (4,M), 594 (10, [M – NO]). Mol.wt., obs.: 594.326, calc. for $C_{34}H_{46}N_2O_7$: 594.331. ^{1}H NMR (270 MHz, $CDCl_3$): δ 2.0 – 2.6 (b), 3.56 (3H,s) 3.94 (9H,s), 4.80 (1H,b), 6.60 (1H,s), 7.60 (1H,b), 7.99 (2H,b). Sample reduced with ascorbate in aqueous methanol: ^{1}H NMR (270 MHz, $CDCl_3$): δ 1.18 (6H,s), 1.22 (6H,s), 1.42 (2H,m), 1.85 (3H,m), 2.02 (2H,m), 2.29 (5H,m), 2.47 (2H,m), 3.54 (3H,s), 3.91 (3H,s), 3.93 (3H,s), 3.95 (3H,s), 4.22 (1H,b), 4.80 (1H,b), 6.21 (2H,b, amido protons), 6.58 (1H,s), 7.58 (1H,d), 7.98 (2H,d).

Cell proliferation. The effect on cell proliferation was tested on cultures of C 1300 mouse neuroblastoma cells (clone 41A3) grown in Ham's F 10 medium supplemented with 10 % newborn calf serum. Cells (5 × 10 cells/dish) were exposed to the colchicine derivatives for 48 h, subsequently detached with trypsin and counted by use of an electronic cell counter. Data were obtained from three separate experiments.

Microtubule proteins and tubulin. Microtubule proteins were prepared from bovine brain by two cycles of assembly – disassembly in the absence of glycerol.[16] Microtuble assembly was monotored continously by the change in absorbance at 350 nm. Assembly was started by increasing the temperature from 0 to 37 °C. The assembly buffer contained 0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid), 1 mM GTP and 0.5 mM $MgSO_4$, pH 6.8. The drugs to be tested were dissolved in ethanol and added to the proteins to a final ethanol concentration of 2 %. The proteins were incubated with the drugs for 10 min at 0 °C before assembly. Tubulin was separated from microtubule associated proteins by phosphocellulose chromatography.[16] The concentration of tubulin was determined from the optical absorption at 278 nm with $A = 1.2$ ml/mg cm and a molecular weight of 110 000 and the concentration of the spin probe and of allocolchicine in ethanol with a millimolar extinction coefficient of 15.9 mM^{-1} cm^{-1} at 291 nm and of colchicine of 17.1 mM^{-1} cm^{-1} at 350 nm.

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