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STEGANACIN: AN INHIBITOR OF HELA CELL GROWTH AND MICROTUBULE ASSEMBLY IN VITRO

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SUMMARY: The plant derivative steganacin, an antitumor compound, blocks the replication of HeLa cells in mitosis. Steganacin inhibits microtubule assembly in vitro (ID_{50} = 1.5 μM) and is a competitive inhibitor of colchicine binding to purified tubulin (K_i = 3.1 μM). The structure-activity relationships of steganacin and a series of analogues are reported.

INTRODUCTION: The lactone steganacin was first studied as an alcoholic extract, prepared from the wood and stems of Steganotaenia araliacea Hochst, and shown to be active against P-388 leukemia in mice, and cytotoxic to cells derived from human carcinoma of the nasopharnyx (1). The total synthesis of steganacin and a series of derivatives has been described (2,3,4).

Steganacin is structurally similar to both colchicine and podophyllotoxin, established inhibitors of microtubule assembly in vitro. All three molecules have a trimethoxybenzene ring. A full description of the stereochemistry of steganacin and its derivatives, episteganacin, steganol, episteganol, steganangin, and isoepisteganacin (an atropisomer of episteganacin) is presented in Fig. 1.

MATERIALS AND METHODS: $Steganacin_a$ and steganangin were kindly provided by the late S. Morris Kupchan and $steganacin_b$, episteganacin, steganol, episteganol, and isoepisteganacin were prepared as previously described (2,3). $Steganacin_a$ is the natural product and $steganacin_b$ the racemic compound. Unless indicated, steganacin refers to the natural product. Podophyllotoxin and vinblastine were obtained from the National Cancer Institute. All drugs were dissolved in dimethyl sulfoxide at a concentration of 10 mM and stored at -20°. The final concentration of dimethyl sulfoxide in each experiment was 0.5%, a concentration that had no effect on control reactions. Fetal calf serum, Joklik's modified Eagle's minimal essential medium and glutamine were purchased from Grand Island Biological Company; [^{3}H]colchicine (19.63 Ci/mmole) and Aquasol from New England

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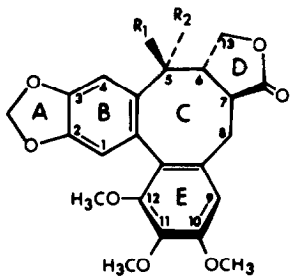
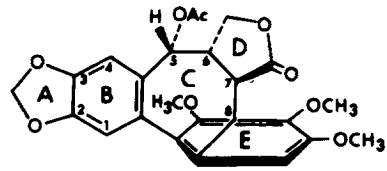
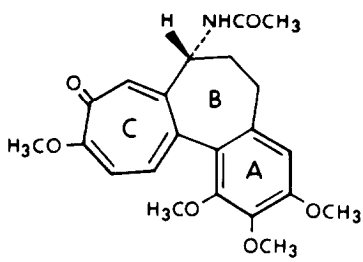


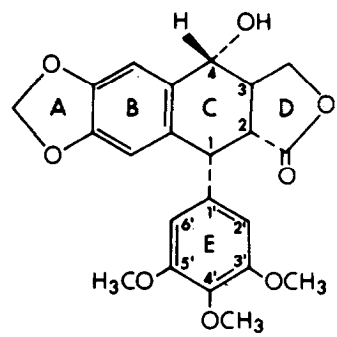


ISOEPISTEGANACIN

DRUG	R_1	R_2	STEREOCHEMISTRY
$Steganacin_a$	OAc	H	Natural product, absolute configuration shown
$Steganacin_b$	OAc	H	Racemic
Episteganacin	H	OAc	Racemic
Steganol	OH	H	Racemic
Episteganol	H	OH	Racemic
Isoepisteganacin	H	OAc	Atropisomer of episteganacin, racemic
Steganangin	O_2C 	H	Natural product, absolute configuration shown



COLCHICINE



PODOPHYLLOTOXIN

Fig. 1. Structural formulae of steganacin, its derivatives, colchicine and podophyllotoxin.

Nuclear and Whatman DE-81 filter discs from Reeve Angel. Fresh chicken heads were obtained from the Jackson Avenue Live Poultry Market, Bronx, New York.

HeLa Cell Culture - HeLa cells, strain S_3, were grown as suspension cultures in Joklik's modified Eagle's minimal essential medium (5) supplemented with 1% glutamine and 5% fetal calf serum. Cell viability was determined by exclusion of trypan blue (6).

Mitotic Index - Five ml of cell suspension was mixed with an equal volume of ethanol: acetic acid: H_2O (5:2:3), centrifuged and resuspended in 0.5 ml of 0.025% crystal violet in 1% acetic acid. Cells in mitosis were determined by counting a minimum of 200 cells and applying the following formula:

$$\text{mitotic index} = \frac{\text{number of cells in mitosis}}{\text{total number of cells}} \times 100\%$$

Purification of Tubulin - Chicken brain tubulin was prepared using two cycles of assembly-disassembly as described by Shelanski et al. (7). Tubulin purified by this method was 85% pure as determined on 7.5% polyacrylamide SDS reducing gels. In binding experiments tubulin concentration was corrected for purity. The purified tubulin was stored at -20° in MES buffer (0.1 M (2 [N-morpholino]ethane sulfonic acid) pH 6.6, containing 1 mM EGTA and 0.5 mM $MgCl_2$) plus 4 M glycerol and 1 mM GTP. Before each experiment, the stored tubulin was dialyzed for 3 hr at 4° against 100 volumes of MES buffer and centrifuged at 120,000 x g for 20 min at 4°. The supernatant, which contains tubulin, was stored in an ice bath until ready for use. All experiments were carried out on tubulin stored for less than two weeks. The method of Lowry et al. (8) was used to determine protein concentration. Bovine serum albumin was used as a standard.

Polymerization of Tubulin - The conditions for the polymerization of tubulin into microtubules have been described by Weisenberg (9). Microtubule assembly was monitored by the method of Gaskin et al. (10). The cuvettes (1 cm path) containing 0.75 ml buffer, drug, and 1 mM GTP were kept at room temperature before adding 1 mg (0.25 ml) tubulin and shifting to 37°. Turbidity measurements were made every 20 sec at 350 nm on a Gilford spectrophotometer equipped with an automatic recorder and a thermostatically regulated liquid circulator.

Electron Microscopy - Microtubule assembly or inhibition of assembly was confirmed by electron microscopy. The negative staining procedure of Bryan was used (11). Samples were examined at 8, 40, and 80 thousand magnifications.

Colchicine Binding Assay - The binding of [^{3}H]colchicine to chicken brain tubulin was measured by a modification of the DEAE filter assay developed by Weisenberg et al. (12). Tubulin in 1 ml MES buffer (pH 6.6) was incubated at 37° with 0.38 - 50.3 μM [^{3}H]colchicine (specific activity, 0.1 Ci/mmole). The reaction was stopped by the addition of 1 ml of 10^{-4} M colchicine. Bound colchicine was determined by filtration under gravity through Whatman DE-81 filter paper (2.4 cm diameter). The filters were washed eight times with 8 ml aliquots of cold 10-fold diluted MES buffer and counted in 10 ml of Aquasol.

RESULTS: The effect of steganacin and its derivatives on mitotic index and cell growth. The effect of steganacin and its derivatives on mitotic index and cell number was analyzed 24 hrs after the addition of the drugs to HeLa cells (Table 1). Steganacin was the most potent inhibitor of cell growth and increased the mitotic index to 94%. At a concentration of 10 μM, steganacin$_a$, steganacin$_b$, steganangin, and steganol were all slightly more potent than colchicine and podophyllotoxin. Episteganol had some activity, while episteganacin and isoepisteganacin had no effect on cell growth. Twenty-four hours after addition of each drug, 98% of the cells were viable as determined by trypan blue exclusion.

Table 1

Effect of Steganacin and its Derivatives

on Mitotic Index and Growth of HeLa Cells

Drug	Mitotic Index (%)	Cell Number/ml(x 10^{-5})
Control	2	4.3
$Steganacin_a$	94	2.0
$Steganacin_b$	89	2.1
Steganangin	86	2.4
Episteganacin	3	4.2
Steganol	92	2.5
Episteganol	28	3.3
Isoepisteganacin	2	4.2
Colchicine	81	2.4
Podophyllotoxin	84	2.4

Cells (2 x 10^5/ml) were incubated at 37° in the presence of 10 μM drug. After 24 hr the mitotic index was determined as described in Materials and Methods and the cell count determined with a hemocytometer.

Effect of steganacin and its derivatives on microtubule assembly in vitro. Turbidity measurements were used to monitor microtubule assembly in vitro. The turbidity developed during microtubule assembly is relatively insensitive to the length of the microtubules formed and is mainly a function of the total mass of supramolecular structures present (10). For this reason electron microscopy was used to confirm all turbidy measurements.

Fig. 2A shows the results of turbidity experiments done with two concentrations of steganacin. At 30 min, 0.39 μM steganacin inhibited assembly by 36% and 3.9 μM by 86%. A log dose response curve for steganacin inhibition of microtubule assembly is given in Fig. 2B. The ID_{50} for steganacin and its derivatives are shown in Table 2. Inhibition greater than 90% was never observed with steganacin. This residual turbidity, seen at concentratipns greater than 3.0 μM drug, is probably due to the small protofilament-like structures observable with the electron microscope at 30 min.

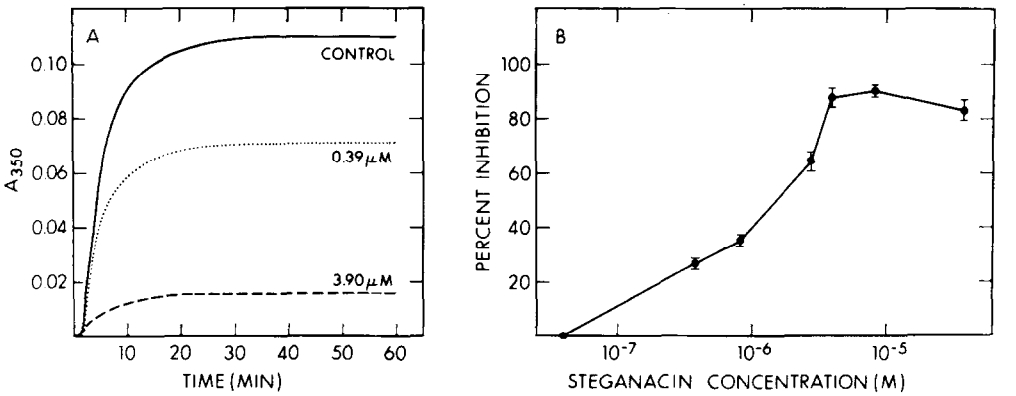


Fig. 2. Effect of steganacin on microtubule assembly. Panel A shows a typical turbidity experiment where A_{350} is plotted as a function of time in the presence of 0.39 (•••) and 3.9 (- -) μM steganacin. Conditions for assembly were 1 mg/ml tubulin, MES buffer, 1 mM GTP, 37°. Panel B shows percent inhibition of microtubule assembly at 30 min as a function of the concentration of steganacin.

Inhibition of colchicine binding by steganacin and its derivatives. The kinetics of colchicine binding to purified tubulin has been described (12,13). Fig. 3 shows the time dependence of [^{3}H]colchicine binding to 1 mg/ml tubulin. Maximum binding was observed at 60 min; thereafter the amount bound decreased. In the presence of 50 μM steganacin or 50 μM podophyllotoxin, colchicine binding was inhibited 47 and 88%, respectively at 60 min. Each of the steganacin derivatives was tested for its ability to alter colchicine binding (Table 2). Those drugs that inhibited [^{3}H]colchicine binding also prevented the decreased binding after 60 min as observed with steganacin and podophyllotoxin (Fig. 3).

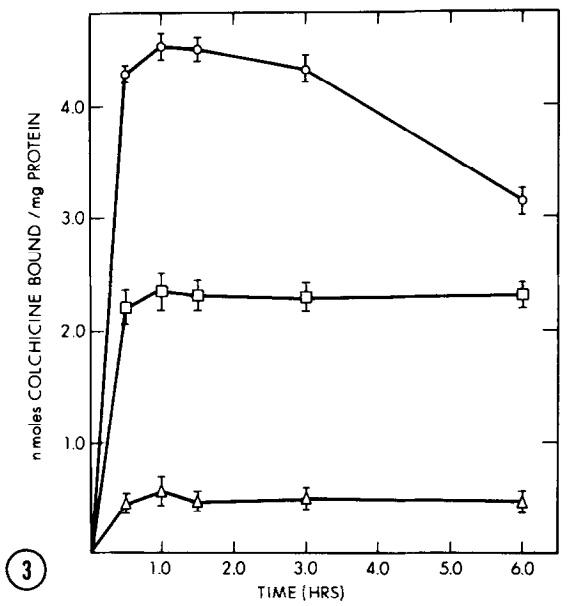
Double-reciprocal plots of [^{3}H]colchicine binding to tubulin in the presence of vinblastine, podophyllotoxin, and steganacin are given in Fig. 4. The apparent affinity (half-maximal binding) of [^{3}H]colchicine for tubulin was calculated to be 2.11 μM. The inhibition constants for steganacin$_a$, steganacin$_b$, steganangin, episteganacin, steganol, and podophyllotoxin relative to [^{3}H]colchicine binding to tubulin are given in Table 2. The inhibition constant (K_i) is assumed to be analogous to the dissociation constant for the

Table 2

Effect of Steganacin and its Derivatives on Microtubule Assembly In Vitro and on Relative Affinity for the Colchicine Binding Site on Tubulin

Drug	Turbidity		Colchicine Binding		
	Inhibition (%)	ID_{50} (μM)	Inhibition (%)	K_i (μM)	Apparent Affinity CLC / K_i drug
$Steganacin_a$	84	1.5	47	3.05	0.691
$Steganacin_b$	52	2.4	45	4.25	0.496
Steganangin	100	1.5	21	8.59	0.245
Episteganacin	23	-	8	135.0	0.016
Steganol	25	-	6	374.0	0.006
Episteganol	0	-	0	-	-
Isoepisteganacin	2	-	2	-	-
Podophyllotoxin	97	0.6	88	1.46	1.443

Turbidity was measured at 30 min in the presence of 50 μM drug. K_i values were determined for 6 drugs. Concentrations chosen were: $steganacin_a$, 1 and 5 μM; $steganacin_b$, 5 μM; steganangin, 5 μM; episteganacin, 50 μM; steganol, 50 μM; and podophyllotoxin, 2 μM. The apparent affinity of colchicine for tubulin is 2.11 μM.



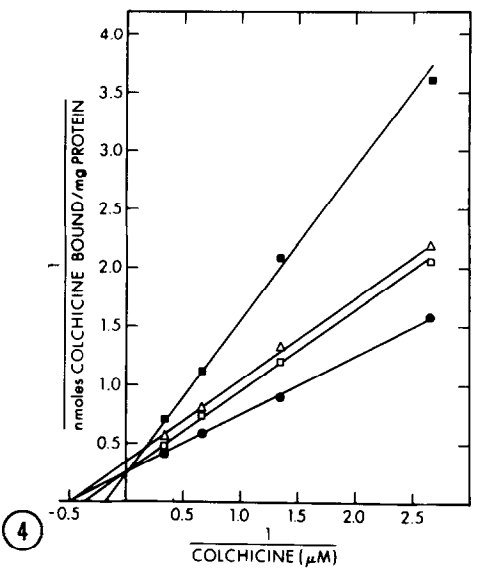


Fig. 3. Binding of 50.3 μM [^{3}H]colchicine to 1 mg/ml tubulin (7.4 μM corrected for purity) at 37° as a function of time. Control (o-o); 50 μM steganacin (□-□); 50 μM podophyllotoxin (Δ-Δ).

Fig. 4. Double-reciprocal plot of steganacin inhibition of [^{3}H]colchicine binding. The binding of [^{3}H]colchicine was done in the absence (●-●) and presence of 1 μM (□-□) and 5 μM (■-■) steganacin and 50 μM vinblastine (Δ-Δ). Each point represents the average of at least three experimental values that did not differ more than 7%. Incubations were for 1 hr at 37° in the presence of 1 mg tubulin at four different [^{3}H]colchicine concentrations (0.38, 0.75, 1.5 and 3 μM) in a final volume of 1 ml. The apparent affinity of [^{3}H]colchicine for tubulin and the K_i values for the inhibitors were computed from the least-squares linear regression lines of the double-reciprocal plots.

drug (14). The K_i for vinblastine, a non-competitive inhibitor under these conditions (15) is 27.2 μM. Both podophyllotoxin and steganacin were found to be competitive inhibitors; podophyllotoxin has a K_i of 1.44 μM and steganacin of 3.05 μM (Table 2). The K_i's for podophyllotoxin and vinblastine were in good agreement with those reported in the literature (16).

DISCUSSION: It has been reported by Wang et al. (17) that steganacin inhibits cleavage of sea urchin eggs. In vitro, it inhibits calf and rabbit tubulin polymerization and causes a slow depolymerization of existing microtubules. We report that steganacin blocks HeLa cell replication in mitosis (mitotic

index of approximately 94% at 10 μM), inhibits chicken brain microtubule assembly in vitro (ID_{50} = 1.5 μM), and competitively inhibits colchicine binding to purified tubulin (K_i = 3.05 μM).

Steganacin$_a$, the natural product was the most potent of the steganacin compounds tested. Steganacin$_b$, a racemic compound was slightly less potent. Steganangin also had strong inhibitory activity. While the above compounds are very close in overall activity, the minor differences between them are reproducible Steganol was not a very active inhibitor of microtubule assembly in vitro or of [^{3}H]colchicine binding. However, steganol is a good inhibitor of cell growth and blocks cells in mitosis. This discrepancy has also been observed with steganol in sea urchin eggs (17), and could be due to the metabolism of steganol to a more active drug in cells, or to a preferential uptake of steganol by cells. Episteganacin had no effect on the mitotic index of HeLa cells, but did demonstrate some minor activity in inhibiting microtubule assembly in vitro and [^{3}H]colchicine binding to purified tubulin. Episteganol was also much less active than steganol. Our data points to the importance of the stereochemistry and substituents at position 5 in ring C for biological activity. Isoepisteganacin, the atropisomer of episteganacin, had no activity in any of the three systems. With the exception of steganol, there was good correlation between the effects of the drugs on cells and on microtubule assembly in vitro.

Colchicine (18), podophyllotoxin (18), and steganangin (19) have been reported to inhibit nucleoside transport in HeLa cells. Steganacin (50 μM) also inhibits the uptake of [^{14}C]uridine in HeLa cells by 65% at 4°. Inhibition is seen as early as 2 min after addition of the drug (data not shown).

A 2-3 fold enhancement of tropolone fluorescence upon interaction of tropolone with tubulin has been described (20). It has been reported that podophyllotoxin does not affect this increase in fluorescence (21). We conducted experiments to determine if steganacin would alter the tropolone fluorescence. We did not observe an enhancement of fluorescence when tropolone

was incubated with our tubulin preparation. The only enhancement of fluorescence we observed was due to light scattering.

Steganacin poisons microtubule assembly *in vitro* in a substoichiometric manner. The ID_{50} for steganacin is 1.5 μM, although the tubulin concentration is 7.4 μM. The mechanism of this poisoning is probably similar to that reported for colchicine (22), since steganacin is a competitive inhibitor of [^{3}H]colchicine binding to tubulin. The inhibition of microtubule assembly *in vitro* by steganacin most likely explains the drugs ability to block HeLa cells in mitosis. These properties may be responsible for the observed antitumor activity of the drug.

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REFERENCES:

1. Kupchan, S.M., Britton, R.W., Ziegler, M.F., Gilmore, C.J., Restivo, R.J., and Bryan, R.F. (1973), J. Amer. Chem. Soc. 95, 1335-1336.
2. Kende, A.S., and Liebeskind, L.S. (1976), J. Amer. Chem. Soc. 98, 267-268.
3. Kende, A.S., Liebeskind, L.S., Kubiak, C., and Eisenberg, R. (1976), J. Amer. Chem. Soc. 98, 6389-6391.
4. Hughes, L.R. and Raphael, R.A. (1976), Tetrahedron Lett. 18, 1543-1546.
5. Grand Island Biological Company (1978), Gibco Catalogue, pp. 130-131, Grand Island, New York.
6. Merchant, D.J., Kahn, R.H., and Murphy, W.H. (1964) Handbook of Cell and Organ Culture, p. 157, Burgess Publishing Company, Minneapolis, Minnesota.
7. Shelanski, M.L., Gaskin, F., and Cantor, C.R. (1973), Proc. Natl. Acad. Sci. USA 70, 765-768.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951), J. Biol. Chem. 193, 265-275.
9. Weisenberg, R.C. (1972), Science 177, 1104-1105.
10. Gaskin, F., Cantor, C.R., and Shelanski, M.L. (1974), J. Mol. Biol. 89, 737-758.
11. Bryan, J. (1976), J. Cell Biol. 71, 749-767.
12. Weisenberg, R.C., Borisy, G.G., and Taylor, E.W. (1968), Biochemistry 7, 4466-4479.
13. Wilson, L. (1970), Biochemistry 9, 4999-5007.
14. Wilson, L., Bamburg, J.R., Mizel, S.B., Grisham, L.M., and Creswell, K.M. (1974), Fed. Proc. 33, 158-166.
15. Zweig, M.H., and Chignell, C.F. (1973), Biochem. Pharmacol. 22, 2141-2150.
16. Snyder, J.A., and McIntosh, J.R. (1976), Ann. Rev. Biochem. 45, 699-720.
17. Wang, R.W., Rebhun, L.I., and Kupchan, S.M. (1977), Cancer Res. 37, 3071-3079.

18. Mizel, S.B., and Wilson, L. (1972), Biochemistry 11, 2573-2578.
19. Horwitz, S.B. (1974), Fed. Proc. 33, 2281-2287.
20. Bhattacharyya, B., and Wolff, J. (1974), Proc. Natl. Acad. Sci. USA 71, 2627-2631.
21. Cortese, F., Bhattacharyya, B., and Wolff, J. (1977), J. Biol. Chem. 252, 1134-1140.
22. Margolis, R.L., and Wilson, L. (1977), Proc. Natl. Acad. Sci. USA 74, 3466-3470.