Inhibition of Tubulin Polymerization by 5,6-Dihydroindolo[2,1-*a*]isoquinoline Derivatives

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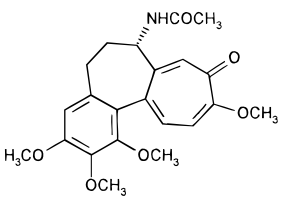
6-Alkyl-12-formyl-5,6-dihydroindolo[2,1-*a*]isoquinolines have been shown to inhibit the growth of human mammary carcinoma cells by an unknown mode of action. One of the possible molecular targets is the tubulin system which is involved in cell division. A number of 5,6-dihydroindolo[2,1-*a*]isoquinolines with methoxy or hydroxy groups in positions 3, 9, and/or 10 and various functional groups such as formyl, acetyl, cyano, alkylimino, and alkylamino in position 12 were synthesized and evaluated for both inhibition of tubulin polymerization and cytostatic activity in MDA-MB 231 and MCF-7 human breast cancer cells. In the tubulin polymerization assay, only hydroxy derivatives were active, whereas both the hydroxy derivatives and some of the methoxy compounds inhibited cell growth. In order to establish a correlation between the inhibition of tubulin polymerization and cytostatic activity in the hydroxy series, two of the most active racemates were separated into the enantiomers. In both assays, the relative potencies of the hydroxy derivatives were in a similar order. Highest activity was found for the (+)-isomers of 6-propyl- (**6b**) and 6-butyl-12-formyl-5,6-hydro-3,9-dihydroxyindolo[2,1-*a*]isoquinoline (**6c**) with IC_{50} values of 11 ± 0.4 and 3.1 ± 0.4 μM, respectively, for the polymerization of tubulin at 37 °C (colchicine: 2.1 ± 0.1 μM). The active hydroxy derivatives displaced 40−70% of [^{3}H]colchicine from its binding site in the tubulin at concentrations 10-fold higher than that of colchicine. The data suggest that hydroxy-substituted indolo[2,1-*a*]isoquinolines bind to the colchicine-binding site and inhibit the polymerization of tubulin. This action can be assumed to be responsible for the cytostatic activity of the hydroxy derivatives and might also contribute to the antitumor effect of the corresponding methyl ethers.

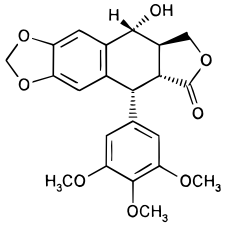
In a previous paper we reported on the cytostatic activities and endocrine properties of a series of acetoxy-substituted 6-alkyl-12-formyl-5,6-dihydroindolo[2,1-*a*]-isoquinolines.[1] Although these compounds bind to the estrogen receptor (ER), they did not show a preference for ER-positive cells when tested for cytostatic activity. IC_{50} values obtained with estrogen-sensitive human MCF-7 breast cancer cells were rather similar to those obtained with hormone-independent MDA-MB 231 mammary carcinoma cells. One of the interesting features of these derivatives was the stereospecificity of the antitumor effect, which was mainly associated with the dextrorotary form.[1] This specific activity prompted us to search for the molecular targets of these agents. In our early studies we failed to demonstrate an interaction with the DNA, the most prominent target molecule for cytostatic drugs. Other cellular structures of interest comprise the signal transduction machinery and the tubulin system. Preliminary studies with EGF-receptor-associated protein tyrosin kinases (PTK) revealed no activity.

The structures of the compounds which inhibit tubulin polymerization exhibit a great diversity which suggests different binding sites at the dimer formed by α- and β-tubulin. The only well-defined pharmacophor for the inhibition of tubulin polymerization is the 3,4,5-trimethoxyphenyl group linked to another aromatic system.[2−9] It is found in colchicin,[10] podophyllotoxin,[11] steganacin,[12] and—in its simplest form—combretastatin A-4[13] (Chart 1). Other simple molecules comprise heterocyclic systems such as 2-phenylquinolinone,[14,15]

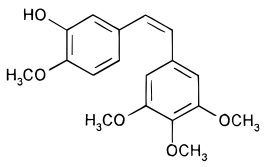
Chart 1. Inhibitors of Tubulin Polymerization



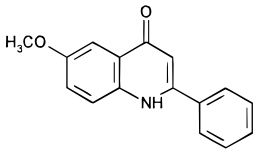
Colchicine



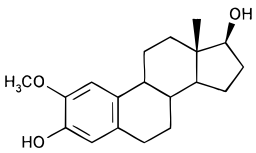
Podophyllotoxin



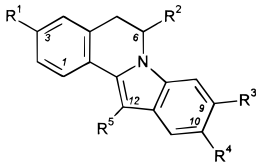
Combretastatin A-4



6-Methoxy-2-phenyl-4-quinolone



2-Methoxyestradiol



R^1 = OH, OCH_3
R^2 = alkyl
R^3, R^4 = H, OH, OCH_3
R^5 = H, CHO, $COCH_3$, CH=NR', CH_2NHR', CN

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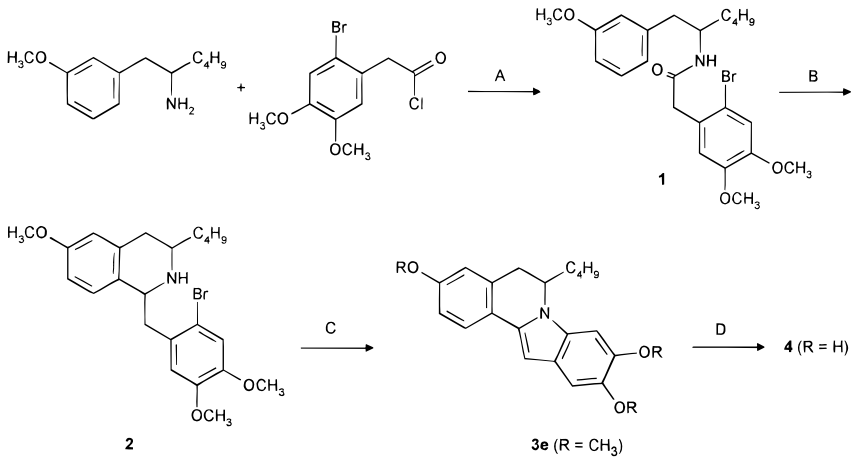
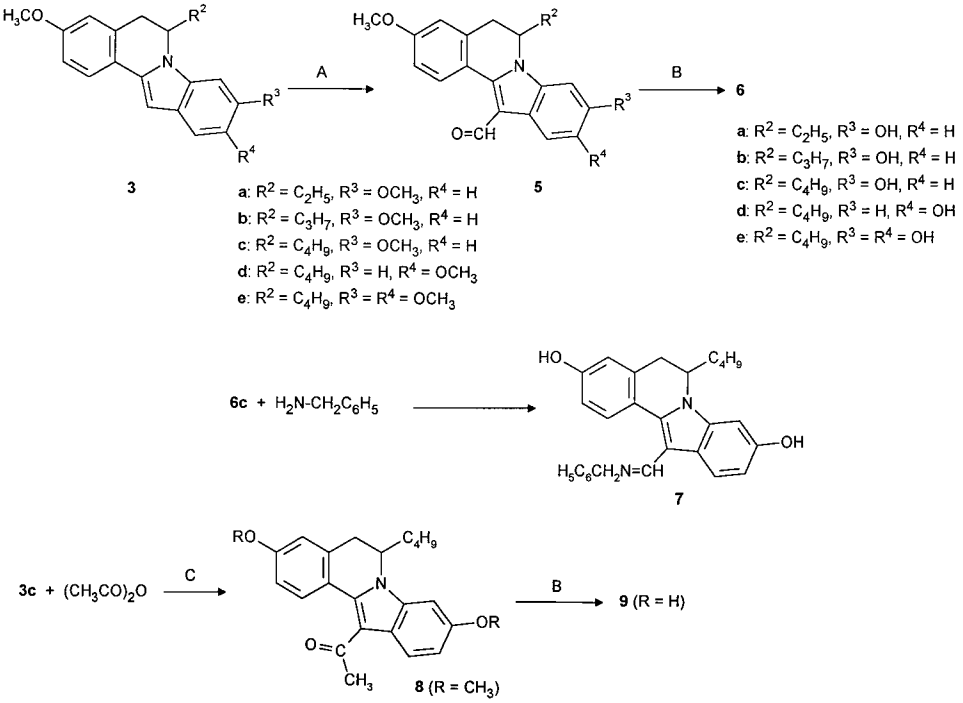
Scheme 1[a]



[a] (A) NEt_3; (B) 1. $POCl_3$, 2. NaOH, 3. $NaBH_4$; (C) 1. NaH/DMSO, 2. Pd/C (10%); (D) BBr_3.

Scheme 2[a]



[a] (A) $POCl_3$/DMF; (B) BBr_3; (C) I_2.

2-styrylquinazolin-4-one,[16,17] 1-deaza-7,8-dihydropteridine,[18] and steroids such as estramustine and 2-methoxyestradiol.[19,20] Different binding sites have to be assumed for the complex structures of the vinca alkaloids[21] and maitansin,[22] though an overlap might be possible. The knowledge that estradiol disrupts microtubuli[23] and the known inhibitory effect of estradiol derivatives on tubulin polymerization[19,20] prompted us to consider an interference with the formation of microtubuli as a possible mode of cytostatic action for the indoloisoquinolines.

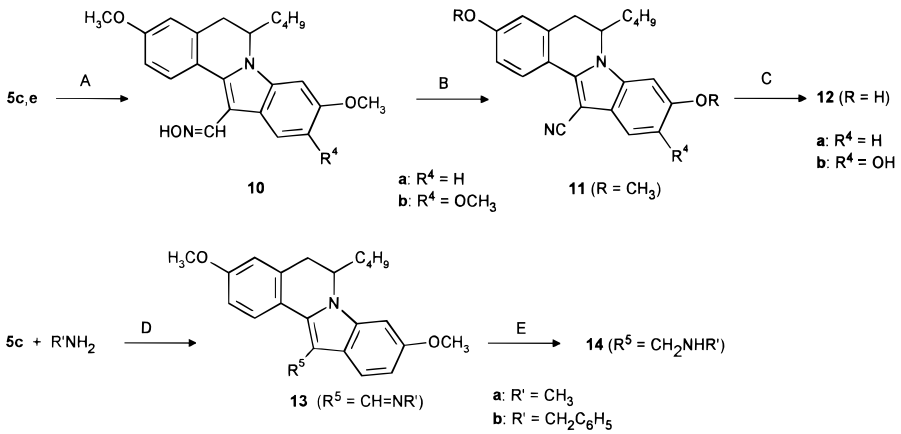
An appropriate method for studying the effect of drugs on polymerization and depolymerization of tubulin is turbidimetry. Microtubuli and α/β-tubulin dimers form a dynamic temperature-dependent equilibrium with preference for the polymer at 37 °C. The concentration of polymer can be measured spectroscopically due to the reflection of light by the microtubuli. In this study we compared the inhibition of tubulin polymerization at 37 °C with the cytostatic activity of a variety of C-12-modified indolo[2,1-*a*]isoquinolines (Chart 1) in two human breast cancer cell lines. The most active compounds were analyzed for their ability to displace colchicine from its binding site.

Chemistry

The route to the tetracyclic skeleton of the 5,6-dihydroindolo[2,1-*a*]isoquinoline, as outlined in Scheme 1, has been described previously.[1] For this study only derivatives with an oxygen function in position 3 and one or two oxygen functions in the indole part (positions 9 and/or 10) were considered. These derivatives have been shown to exert the strongest cytostatic effects when a propyl or butyl group was linked to position 6.

The structural variations presented here concerned the functional group in position 12 and the conversion of the methoxy derivatives into the free phenols (Scheme 2). The formyl group in 6-butyl-12-formyl-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (**5c**) and 6-butyl-12-formyl-5,6-dihydro-3,9,10-trimethoxyindolo[2,1-*a*]i-

Scheme 3[a]



[a] (A) $HONH_3Cl/Na_2CO_3$; (B) Ac_2O/pyridine; (C) BBr_3; (D) HOAc; (E) $NaBH_4$.

soquinoline (**5e**), respectively, was converted into the nitrile (**11a**,**b**) via the oxime (**10a**,**b**).

Compound **5c** reacted with methylamine and benzylamine to produce the respective imines **13a**,**b**, which gave the corresponding amines **14a**,**b** upon treatment with sodium boron hydride (Scheme 3). As an alternative to the formyl group, the acetyl group was introduced into the indolo[2,1-*a*]isoquinoline system by treatment with acetic acid anhydride in the presence of iodine to give the 12-acetyl derivative **8**. All of the methoxy compounds except the imines **13** and amines **14** were converted to the free phenols by treating them with boron tribromide. Direct reaction of the phenolic aldehyde **6c** with benzylamine led to the imine **7** with unprotected hydroxy groups.

Biological Results and Discussion

The aim of this study was the identification of the molecular target which is involved in the cytostatic action of 12-substituted 5,6-dihydroindolo[2,1-*a*]isoquinolines. Since preliminary investigations revealed neither an interaction with the DNA nor an interference with the signal transduction machinery, we considered the tubulin or its polymeric structure, the microtubuli, as the potential site of action. The tubulin used in this study was isolated from fresh calf brains by several centrifugation steps at two different temperatures: 37 °C to polymerize tubulin to microtubuli and 2 °C for depolymerization. Two cycles of polymerization/depolymerization were usually sufficient to get electrophoretically pure tubulin that was still associated with MAPs (microtubuli-associated proteins). In the initial experiments, all compounds of this study were evaluated for their inhibitory activity on tubulin polymerization in a standard concentration of 40 μM (Table 1). Strong inhibition was only observed for the hydroxy derivatives but not for the corresponding methyl ethers. Among the free phenols, the 12-unsubstituted tetracycle **4**, the 6-ethyl derivative **6a,** and the 12-acetyl compound **9** were only weak inhibitors.

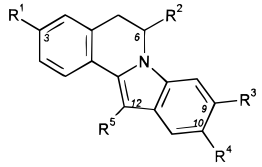
Both methoxy derivatives and free phenolic compounds were tested for cytostatic activity in human MDA-MB 231 breast cancer cells which lack estrogen receptors. In this assay the methoxy compounds inhibited cellular growth to a varying degree. Lowest IC_{50} values were found for both nitriles (**11a**,**b** $\sim$ 3 μM) and the methylamino derivative **14a** (2.1 μM) (Table 1). The cytostatic activities of the phenolic compounds exceeded those of the corresponding methyl ethers. When the dose−response curves of some of the more potent hydroxy derivatives were plotted, deviations from the sigmoid curve were observed with a more or less pronounced minimum in the submicromolar range and the normal decline above 1 μM. In the case of **6b** and **12a** the minimum was as low as 25−30% T/C at 5 × 10^{-7} M. This biphasic course of the curve was also found for 2-methoxyestradiol and was confirmed in additional experiments.

When the cytostatic effects of the hydroxy derivatives were compared with the inhibition of tubulin polymerization, some agreement was observed though the concentration in the tubulin polymerization assay was considerably higher. The discrepancy between concentrations required for cytostatic action and those for the inhibition of tubulin polymerization has been noticed in many other classes of antimitotic agents.[4,7,8,24] The most active hydroxy compounds were the formyl derivatives **6b**,**d** and the nitrile **12a** with IC_{50} values below 10^{-6} M. The benzylimine of **6c** (**7**) showed an inhibitory effect similar to that of the parent compound which might be due to a hydrolysis reaction under the conditions of the assay.

Similar results were obtained with estrogen-sensitive MCF-7 breast cancer cells which were used as an additional model for the evaluation of the phenolic derivatives because of their affinity for the estrogen receptor. The relative binding affinities for the calf uterine estrogen receptor were in the range of 0.5−2.0% of estradiol. At a concentration of 1 μM, the 6-ethyl (**6a**) and the 12-acetyl (**9**) derivatives as well as the trihydroxy compounds **6e** and **12b** were inactive, whereas all of the other hydroxy derivatives completely blocked cell proliferation. For the majority of the tested compounds no marked differences between both cell lines were detected. Thus, an estrogen receptor-mediated action in the estrogen receptor-positive MCF-7 cells is unlikely.

In a previous paper,[1] we reported on the stereospecificity of the antitumor effect of the acetates of **6b**,**c**. This observation prompted us to separate the racemic mixtures of the hydroxy derivatives **6b**,**c** by liquid chromatography to obtain the pure enantiomers (+)-**6b**, (−)-**6b** and (+)-**6c**, (−)-**6c**, respectively. These compounds together with the racemates and the racemic mixtures of **6d**,**e**, and **12a**,**b** were tested at several concentrations

Table 1. Effect of Various Methoxy- and Hydroxy-Substituted 6-Alkyl-5,6-dihydroindolo[2,1-*a*]isoquinolines on Tubulin Polymerization and the Growth of Human Mammary Carcinoma Cells



							IC_{50} [μM]	
compd	R^1	R^2	R^3	R^4	R^5	ITP[a] (%)	MDA[b]	MCF-7[c]
3e	OCH_3	*n*-C_4H_9	OCH_3	OCH_3	H	41	>10	
5b	OCH_3	*n*-C_3H_7	OCH_3	H	CHO	17	>10	
5c	OCH_3	*n*-C_4H_9	OCH_3	H	CHO	11	8.7	
5d	OCH_3	*n*-C_4H_9	H	OCH_3	CHO	10	>10	
5e	OCH_3	*n*-C_4H_9	OCH_3	OCH_3	CHO	1	9.0	
8	OCH_3	*n*-C_4H_9	OCH_3	H	$COCH_3$	5	>10	
10a	OCH_3	*n*-C_4H_9	OCH_3	H	CH=NOH	17	>10	
11a	OCH_3	*n*-C_4H_9	OCH_3	H	CN	16	3.1	
11b	OCH_3	*n*-C_4H_9	OCH_3	OCH_3	CN	32	2.7	
13a	OCH_3	*n*-C_4H_9	OCH_3	H	$CH=NCH_3$	3	7.8	
13b	OCH_3	*n*-C_4H_9	OCH_3	H	$CH=NCH_2C_6H_5$	16	5.7	
14a	OCH_3	*n*-C_4H_9	OCH_3	H	CH_2NHCH_3	9	2.1	
14b	OCH_3	*n*-C_4H_9	OCH_3	H	$CH_2NHCH_2C_6H_5$	27	7.0	
4	OH	*n*-C_4H_9	OH	OH	H	33	>10	>10
6a	OH	C_2H_5	OH	H	CHO	11	2.8	2.5
6b	OH	*n*-C_3H_7	OH	H	CHO	93	0.3	0.29
6c	OH	*n*-C_4H_9	OH	H	CHO	100	1.4	0.22
6d	OH	*n*-C_4H_9	H	OH	CHO	81	0.2	0.65
6e	OH	*n*-C_4H_9	OH	OH	CHO	100	3.2	>10
7	OH	*n*-C_4H_9	OH	H	$CH=NCH_2C_6H_5$	93	2.1	0.26
9	OH	*n*-C_4H_9	OH	H	$COCH_3$	24	2.2	2.5
12a	OH	*n*-C_4H_9	OH	H	CN	100	0.3	0.54
12b	OH	*n*-C_4H_9	OH	OH	CN	79	3.4	2.7
colchicine						100	0.03	
tamoxifen								0.15

[a] Inhibition of tubulin polymerization by a 40 μM solution of test compounds, measured after 20 min at 37 °C. Mean values of two independent experiments. Full details are presented in the text. [b] Inhibition of the growth of MDA-MB 231 human breast cancer cells after incubation for 4 days, determined by measuring optical densities after crystal violet staining of vital cells. [c] Inhibition of the growth of estrogen-sensitive MCF-7 human breast cancer cells after incubation for 8 days, determined by measuring optical densities after crystal violet staining of vital cells. Only derivatives with free hydroxy groups and tamoxifen as reference drug were tested.

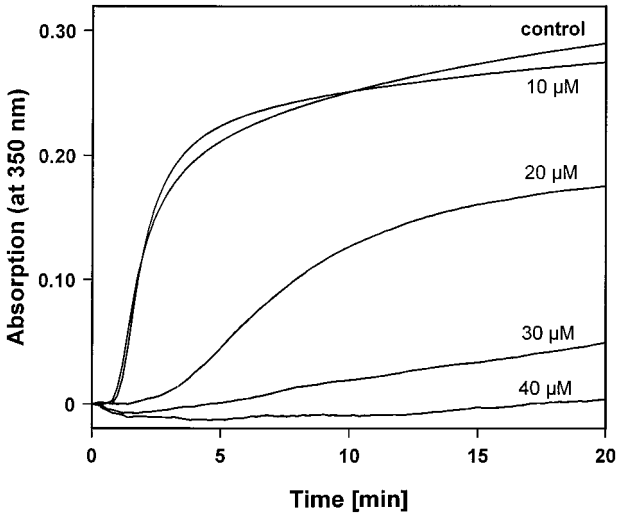


Figure 1. Effect of various concentrations of 5,6-dihydroindolo[2,1-*a*]isoquinoline **6e** on the polymerization of calf brain tubulin. UV absorption was recorded for 20 min after the temperature had been switched from 2 to 37 °C. Control curve represents unaffected tubulin polymerization.

for their inhibitory effect on tubulin polymerization (Figure 1, Table 2). Characteristic polymerization curves were obtained for all of the compounds with plateaus on different levels (Figure 1). From the calculated data it became evident that mainly or even exclusively the (+)-isomers with IC_{50} values of 11 and 3.1 μM, respectively, were responsible for the inhibition of tubulin polymerization of the racemates **6b**,**c**. The value for (+)-**6c** was close to those of the reference drugs colchicine (2.1 μM) and podophyllotoxin (1.2 μM). The differences in activity between the enantiomers were paralleled by the results obtained with the acetates in MDA-MB 231 cells. The lack of sufficient quantities of the pure enantiomers of the phenols did not allow us to use the free hydroxy derivatives for the determination of cytostatic activity. Significant differences in cytostatic activity between hydroxy derivatives and their actetates however have not been noted for several 5,6-dihydroindolo[2,1-*a*]isoquinolines (data not shown) or 2-phenylindoles[25] when both types of compounds were tested *in vitro* for growth inhibition of MDA-MB 231 and MCF-7 cells, respectively. The values for the reference drugs colchicine and podophyllotoxin were close to those reported in the literature for their inhibitory effects on tubulin polymerization (colchicine 1.9;[14] podophyllotoxin 2.1 ± 0.1,[7] 1.3 ± 0.6[14]).

Inhibition of tubulin polymerization can be considered as the consequence of the binding interaction with the α/β-tubulin dimer though other mechanisms such as interactions with magnesium ions or GTP are conceivable. One of the obvious sites is that at which colchicine and a number of other agents with two aromatic domains bind. Thus, we tested the enantiomers and

Table 2. Inhibitory Effects of Racemic 5,6-Dihydroindolo[2,1-*a*]isoquinolines and Enantiomers on Tubulin Polymerization, Colchicine Binding, and Cell Growth

compd	ITP[a] (IC_{50}, μM, ± SD)	ICB[b] (%)	ICG[c] (IC_{50}, μM)	
			R^1,$R^{3(4)}$ = OH	R^1,R^3 = OAc
(±)-**6b**	19 ± 1 (16 ± 2[d])	40 (33[d])	0.3	0.45[d]
(+)-**6b**	11 ± 0.4	53	nd	0.35[d]
(−)-**6b**	>70	27	nd	5.5[d]
(±)-**6c**	8.9 ± 0.3 (4.9 ± 0.1 at 26 °C)	67	1.4	0.32[d]
(+)-**6c**	3.1 ± 0.4	69[e]	nd	0.15[d]
(−)-**6c**	>60	22	nd	0.78[d]
6d	19 ± 3	nd	0.2	
6e	19 ± 1	57	3.2	
12a	13 ± 2	48	0.3	
12b	25 ± 1	54	3.4	
colchicine	2.1 ± 0.1 (1.7 ± 0.05 at 26 °C)		0.030	
podophyllotoxin	1.2 ± 0.1	89	0.026	
vinblastine	0.7 ± 0.1	−64	0.011	
2-methoxyestradiol	60 ± 2 (12 ± 0.5 at 26 °C)	44	0.25 and ~5[f]	

[a] Inhibition of tubulin polymerization: IC_{50} values were determined after 20 min at 37 °C. Mean values of three independent experiments. Full details are presented in the text. [b] Inhibition of colchicine binding. Reaction mixtures contained tubulin, 1 μM [^{3}H]colchicine, and 10 μM inhibitor and were incubated for 30 min at 37 °C prior to analysis. Mean of two independent experiments. [c] Inhibition of cell growth. The IC_{50} values were determined with MDA-MB 231 breast cancer cells. Mean of two independent experiments. [d] Values of the respective diacetates whose syntheses are described in ref.[1] [e] 30% inhibition for equimolar concentrations (1 μM) of colchicine and inhibitor. [f] Biphasic dose−response curve with maximum inhibition (91%) at 1 and >10 μM.

some of the racemic mixtures for their capability of displacing [^{3}H]colchicine from its binding site. At concentrations 10-fold higher than that of colchicine, the tubulin-bound radioactivity was decreased substantially. The differences between the optical isomers, though also observed in this experiment, were less pronounced than in the other assays. Podophyllotoxin displaced 89% of colchicine from its binding site when dosed 10-fold higher and 69% in equimolar concentration. These values are similar to those reported by other authors.[16] Vinblastine increased the amount of colchicine bound to tubulin due to a stabilization of the binding.[26]

The comparison with 2-methoxyestradiol was of particular interest in this study because this compound shares the binding affinity for the estrogen receptor with the indoloisoquinoline system and has been reported to inhibit tubulin polymerization by interacting with the colchicine-binding site.[19] The latter effect was only marked at suboptimal conditions at a polymerization temperature of 26 °C and was rather small at 37 °C. We made the same observation when we tested 2-methoxyestradiol at both temperatures. The values of other compounds such as colchicine or **6c** were also influenced by the temperature but to a smaller extent (Table 2**)**. Similar results were obtained in a series of diaryl compounds when tested at 30 and 37 °C.[6] The binding affinity of methoxyestradiol for the colchicine-binding site was somewhat lower than that observed for the most active indoloisoquinolines and was consistent with that reported in the literature (34% inhibition versus 38%[27]).

When we determined the cytostatic activity of 2-methoxyestradiol in MDA-MB 231 cells, we noticed a biphasic course of the dose−response curve. Maximum growth inhibition was observed at a concentration of 1 μM. At lower and higher concentrations the inhibitory effect was diminished. Possibly, a protective effect conteracts the inhibition at concentrations between 1 and 10 μM. This unusual reaction of the MDA-MB cells, which was also observed for **6a** and **12a**, was confirmed by repeating these experiments several times.

The investigations reported in this paper were aimed in two directions: the elucidation of the mechanism by which 5,6-dihydroindolo[2,1-*a*]isoquinolines exert their antitumor activity and the improvement of cytostatic potency of this class of compounds by modifying the substituent at C-12. One of the molecular targets that we considered for the action of these compounds was the tubulin system. When we evaluated the effect of various indoloisoquinoline derivatives on the polymerization of tubulin, only hydroxy derivatives proved to be inhibitors with variable potencies whereas none of the methoxy derivatives strongly interfered with the formation of microtubuli. This result was unexpected because most of the known antimitotic compounds are characterized by the presence of aromatic methoxy groups as exemplified by colchicine and combretastatin A-4 (Chart 1). Since the synthetic procedures applied did not allow the preparation of derivatives with only one ether function cleaved, it was not possible to study mixed hydroxy/methoxy compounds. Thus, it remains unclear whether one or two phenolic groups are required for strong inhibition of tubulin polymerizaton.

The structural alterations concerned mainly the substituent at C-12 of the tetracyclic indoloisoquinoline system. The replacement of the formyl group by other functional groups such as acetyl, alkylimino, alkylamino, and cyano improved neither the cytostatic activity nor the inhibitory effect on tubulin polymerization. The alkylimino derivatives appeared to function as prodrugs giving the same results as the parent formyl compound. A second hydroxy group in the indole moiety brought no advantage.

When the cytostatic activities of the hydroxy derivatives were compared with their ability of inhibiting tubulin polymerization, a similar order of relative potencies was observed. The average IC_{50} values for these two effects, however, were quite different. The concentrations for the inhibition of cell growth were 2 orders of magnitude lower than those for the cell-free experiments on tubulin polymerization. This difference however seems to be a general feature of antimitotic drugs because it has been reported in several papers[4,7,8,24,28] and might reflect the differences in the experimental design. In the cellular assay, living cells are treated at 37 °C with the drug, whereas the turbidimetric determinations are performed with a

preparation of isolated tubulin and involve a depolymerization step followed by the temperature-dependent polymerization. It is conceivable that small quantities of an antimitotic compound can interact with a few essential sites in the microtubuli leading to disruption and preventing the formation of the mitotic spindle. Therefore, we have planned to analyze the antibody-labeled microtubuli by fluorescence microscopy in the presence and absence of antimitotic drugs. Maybe, it is possible to detect cellular changes at concentrations significantly lower than those required for inhibition of polymerization.

In this study, we found a distinct difference between the hydroxy-substituted indoloisoquinolines and the methoxy derivatives with cytostatic activity such as the two nitriles and the methylamino derivative. Though compounds of both series possess considerable cytostatic activity, only the phenolic compounds inhibit the polymerization of tubulin under the conditions of the turbidimetry assay. Several reasons can account for this apparent difference: (i) The methoxy derivatives inhibit cellular growth by a mode of action different from that of the phenolic compounds although both series share the same tetracyclic skeleton. (ii) The increased lipophilicity of the methyl ether facilitates the penetration of the compounds into the tumor cells which might increase cytostatic activity. (iii) A different kind of interaction with the tubulin takes place which does not prevent polymerization. An interesting example for the latter effect is curacin A, a newly isolated lipid natural product that binds to the colchicine site of tubulin and inhibits mitosis.[24] It was shown to interfere with the polymerization reaction in a rather unusual manner giving rise only to partial inhibition even at high concentrations.[29] Under these aspects, further studies with different techniques are warranted to clarify whether the methoxy-substituted indolo[2,1-*a*]isoquinolines act via the tubulin system or an alternate mechanism.

Experimental Section

Melting points were determined on a Büchi 510 apparatus and are uncorrected. Elemental analyses were performed by Mikroanalytisches Laboratorium, University of Regensburg, and were within ±40% of the calculated values except where noted. NMR spectra were obtained on a Bruker AC-250 spectrometer with TMS as internal standard and are consistent with the assigned structures. Mass spectra were recorded on a Varian MAT 311A spectrometer. The syntheses of the 6-alkyl-5,6-dihydro-3,9(10)-dimethoxyindolo[2,1-*a*]isoquinolines **3a**−**d** and their 12-formyl derivatives **5a**−**d** have been described previously.[1]

(2-Bromo-4,5-dimethoxyphenyl)-*N*-[1-(3-methoxyphenyl)hex-2-yl]acetamide (1). Under N_2, a solution of 2-bromo-4,5-dimethoxyphenylacetic acid chloride (0.10 mol) in 110 mL of dry CH_2Cl_2 was added dropwise to a stirred solution of 2-amino-1-(3-methoxyphenyl)hexane (0.10 mol) and triethylamine (0.10 mol) in 250 mL of dry CH_2Cl_2. Stirring was continued at room temperature for 1 h. The reaction mixture was poured into ice−water, acidified with 2 N HCl, and extracted with CH_2Cl_2. The combined organic layers were washed with $NaHCO_3$ solution and water and dried (Na_2SO_4). After evaporation of the solvent the crude product was purified by chromatography (SiO_2; CH_2Cl_2/Et_2O, 7:1). The product was crystallized from Et_2O/EtOAc (1:1) to afford colorless crystals (23%): mp 112−114 °C; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, ^3J = 7 Hz, 3H, -CH_2-CH_3), 1.04−1.49 (m, 6H, -(CH_2)$_3$-CH_3), 2.72 (d, ^3J = 7 Hz, 2H, -CH_2-CH-), 3.59 (s, 2H, CO-CH_2-), 3.76 (s, 3H, -OCH_3), 3.84 (s, 3H, -OCH_3), 3.88 (s, 3H, -OCH_3), 4.05−4.22 (m, 1H, -C*H*-NH-), 5.20 (d, br, ^3J = 9 Hz, 1H, -NH-), 6.52−7.26 (m, 4H, Ar-H), 6.75 (s, 1H, Ar-H), 7.01 (s, 1H, Ar-H). Anal. ($C_{23}H_{30}BrNO_4$) C, H, N.

1-(2-Bromo-4,5-dimethoxybenzyl)-3-butyl-1,2,3,4-tetrahydro-6-methoxyisoquinoline (2). Under N_2, 66 mmol of $POCl_3$ was added to a solution of the acetamide **1** (23 mmol) in 35 mL of dry MeCN. After heating for 3 h under reflux, the mixture was poured into ice−water. With cooling, the mixture was made alkaline by addition of 40% aqueous NaOH. The aqueous solution was extracted with CH_2Cl_2. The combined organic layers were washed with water and saline and dried (Na_2SO_4). After evaporation of the solvent, the crude product was dried under high vacuum and dissolved in 100 mL of dry MeOH. After cooling to 0 °C, sodium borohydride (73 mmol) was added in small portions. Stirring was continued at room temperature for 1 h followed by heating under reflux for 1 h. After evaporation of the solvent, CH_2Cl_2 and water were added. The organic layer was separated and the aqueous one extracted with CH_2Cl_2. The combined organic layers were washed with saturated NaCl solution and dried (Na_2SO_4). The residue obtained after evaporation of the solvent was purified by chromatography (SiO_2; CH_2Cl_2/Et_2O, 5:1). The product was obtained as a colorless oil (42%): ^{1}H NMR ($CDCl_3$) δ 0.87 (t, ^3J = 7 Hz, 3H, -CH_2-CH_3), 1.18−1.50 (m, 6H, -(CH_2)$_3$-CH_3), 1.55 (s, br, 1H, -NH-), 2.52−2.79 (m, 4H, -CH_2-C*H*-, -C*H*-H), 3.61, 3.66 (dd, ^2J = 14 Hz, ^3J = 3 Hz, 1H, -CH-*H*), 3.79 (s, 3H, -OCH_3), 3.83 (s, 3H, -OCH_3),3.88(s, 3H,-OCH_3), 4.27 (d, ^3J = 8 Hz, 1H, Ar-C*H*-NH), 6.64 (d, ^3J = 9 Hz, 1H, Ar-H), 6.75, 6.79 (dd, ^3J = 9 Hz, ^4J = 3 Hz, 1H, Ar-H), 6.89 (s, 1H, Ar-H), 7.07 (s, 1H, Ar-H), 7.34 (d, ^3J = 9 Hz, 1H, Ar-H). Anal. ($C_{23}H_{30}BrNO_3$) C, H, N.

6-Butyl-5,6-dihydro-3,9,10-trimethoxyindolo[2,1-*a*]isoquinoline (3e). A solution of (bromobenzyl)tetrahydroisoquinoline **2** (9.5 mmol) in 37 mL of DMSO was added to a solution of sodium (methylsulfinyl)methanide prepared from 67 mmol of NaH (80% in oil dispersion) and 37 mL of DMSO. After stirring for 18 h, the mixture was poured into 300 mL of water containing an excess of NH_4Cl and extracted with CH_2Cl_2. The organic layer was washed with water and saline. After drying (Na_2SO_4) and evaporation of the solvent, an oil was obtained. Chromatography (SiO_2; CH_2Cl_2) yielded a mixture of dihydro- and tetrahydroindoloisoquinolines which was not separated but directly converted into the dihydro derivative.

Under N_2, the mixture of dihydro- and tetrahydroindoloisoquinolines (2.5 g) and Pd/C (10%, 0.90 g) was mixed thoroughly in a round bottom flask, which was then placed in a hot oil bath. The temperature was kept at 120 for 4 h. After cooling, the mixture was dissolved in $CHCl_3$ and filtered. The solvent was evaporated and the residue crystallized from EtOH. After recrystallization colorless crystals (45%) were obtained: mp 137−139 °C; ^{1}H NMR ($CDCl_3$) δ 0.82 (t, ^3J = 7 Hz, 3H, -CH_2-CH_3), 1.19−1.59 (m, 6H, -(CH_2)$_3$-CH_3), 2.93, 2.99 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{AX}$ = 1 Hz, 1H, H_A-5), 3.33, 3.38 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{BX}$ = 6 Hz, 1H, H_B-5), 3.84 (s, 3H, -OCH_3), 3.93 (s, 3H, -OCH_3), 3.96 (s, 3H, -OCH_3), 4.50−4.58 (m, 1H, H-6), 6.62 (s, 1H, H-8), 6.77 (d, ^4J = 2 Hz, 1H, H-4), 6.80 (s, 1H, H-11), 6.81, 6.85 (dd, ^4J = 2 Hz, ^3J = 9 Hz, 1H, H-2), 7.06 (s, 1H, H-12), 7.60 (d, ^3J = 9 Hz, 1H, H-1). Anal. ($C_{23}H_{27}NO_3$) C, H, N.

6-Butyl-5,6-dihydro-3,9,10-trihydroxyindolo[2,1-*a*]isoquinoline (4). Under N_2, 5.5 mmol of BBr_3 in 5 mL of dry CH_2Cl_2 was added slowly to a solution of the trimethoxy derivative **3e** in 15 mL of dry CH_2Cl_2 at −50 °C. After addition, stirring was continued for 30 min at −50 °C and 18 h at room temperature. The mixture was carefully hydrolyzed by adding saturated $NaHCO_3$ solution. After addition of 60 mL of EtOAc, the mixture was vigorously stirred for 15 min. After separation of the layers the aqueous phase was extracted several times with EtOAc. The combined organic layers were washed with water and saline and dried (Na_2SO_4). After evaporation of the solvent in vacuo, the product was purified by chromatography (SiO_2; CH_2Cl_2/Et_2O, 2:1) to give off-white crystals (88%): mp 183−185 °C; ^{1}H NMR ($CDCl_3$) δ 0.81 (t, ^3J = 7 Hz, 3H, -CH_2-CH_3), 1.16−1.51 (m, 6H, -(CH_2)$_3$-CH_3), 2.97, 3.03 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{AX}$ = 1 Hz, 1H, H_A-5), 3.22, 3.29 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{BX}$ = 6 Hz, 1H, H_B-5), 4.55 (m, 1H, H-6), 6.50

(s, 1H, vinyl-H), 6.74 (s, 1H, OH) 6.75 (s, 1H, Ar-H), 6.78 (s, 1H, Ar-H), 6.85 (s, 1H, Ar-H), 7.46 (s, 1H, OH), 7.50−7.63 (m, 2H, Ar-H), 8.39 (s, 1H, OH). Anal. ($C_{20}H_{21}NO_3$) C, H, N.

6-Butyl-12-formyl-5,6-dihydro-3,9,10-trimethoxyindolo[2,1-*a*]isoquinoline (5e). Dry DMF (1.9 mL) was added slowly to 29 mmol of $POCl_3$ at 10−20 °C under N_2. A solution of **3e** (2.9 mmol) in 18 mL of dry DMF was added slowly to keep the temperature below 35 °C. Stirring was continued for 1 h at room temperature. The mixture was then poured into ice−water and made alkaline by addition of NaOH (40%). The solution was extracted with CH_2Cl_2. The organic layer was washed with water and saline and dried (Na_2SO_4). The solvent was removed in vacuo and the residue purified by chromatography (SiO_2; CH_2Cl_2/Et_2O, 5:1) to give a greenish amorphous material (76%): mp 78−80 °C; ^{1}H NMR ($CDCl_3$) δ 0.84 (t, ^3J = 7 Hz, 3H, -CH_2-CH_3), 1.21−1.68 (m, 6H, -(CH_2)$_3$-CH_3), 3.00, 3.02 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{AX}$ = 1 Hz, 1H, H_A-5), 3.33, 3.38 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{BX}$ = 6 Hz, 1H, H_B-5), 3.89 (s, 3H, -OCH_3), 3.97 (s, 3H, -OCH_3), 4.01 (s, 3H, -OCH_3), 4.51−4.59 (m, 1H, H-6), 6.82 (s, 1H, H-8), 6.88 (d, ^4J = 2 Hz, 1H, H-4), 6.91, 6.94 (dd, ^3J = 9 Hz, ^4J = 2 Hz, 1H, H-2), 7.82 (d, ^3J = 9 Hz, 1H, H-1), 7.95 (s, 1H, H-11), 10.41 (s, 1H, -CHO); IR (KBr) 1645 cm^{-1}. Anal. ($C_{24}H_{27}NO_4$) C, H, N.

6-Ethyl-12-formyl-5,6-dihydro-3,9-dihydroxyindolo[2,1-*a*]isoquinoline (6a). Under N_2, 0.35 mL (3.6 mmol) of BBr_3, dissolved in 2 mL of dry CH_2Cl_2, was slowly added to a solution of 6-ethyl-12-formyl-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (**5a**)[1] (1.0 mmol) in 18 mL of dry CH_2Cl_2 at a temperature of −50 °C. After stirring for 30 min at this temperature, the mixture was allowed to warm up to room temperature, and stirring was continued for 15 h. With cooling a saturated solution of $NaHCO_3$ was added until the vigorous reaction ceased. After addition of 60 mL of EtOAc, stirring was continued for 15 min followed by filtration. The layers were separated, and the aqueous layer was extracted three times with EtOAc. After washing with water and drying (Na_2SO_4), the solvent was removed in vacuo and the residue purified by chromatography (SiO_2; $CHCl_3/Et_2O$, 1:1). Crystallization from $CHCl_3$ afforded light-green crystals (83%): dec >215 °C; ^{1}H NMR (DMSO-d_6) $_2$-CH_3), 1.28−1.62 (m, 2H, -CH_2-CH_3), 3.02 (dd, $^2J_{AB}/^3J_{AX}$ = 16/1 Hz, 1H, -C*H*-H, H_A-5), 3.22 (dd, $^2J_{AB}/^3J_{BX}$ = 16/6 Hz, 1H, -CH-*H*, H_B-5), 4.66 (m, 1H, -CH-, H-6), 6.71 (dd, $J_{1/2}$ = 8/2 Hz, 1H, Ar-H), 6.80−6.89 (m, 3H, Ar-H), 7.82 (d, J = 8 Hz, 1H, Ar-H), 7.98 (d, J = 8 Hz, 1H, Ar-H), 9.41 (s, 1H, -OH), 10.06 (s, 1H, -OH), 10.26 (s, 1H, -CHO); IR (KBr) 3320, 1610 cm^{-1}. Anal. ($C_{19}H_{17}NO_3$) C, H, N.

12-Formyl-5,6-dihydro-3,9-dihydroxy-6-propylindolo[2,1-*a*]isoquinoline (6b): prepared in 89% yield from 12-formyl-5,6-dihydro-3,9-dimethoxy-6-propylindolo[2,1-*a*]isoquinoline (**5b**)[1] by a method similar to that described for **6a**; light-green crystals; dec >225 °C; ^{1}H NMR (DMSO-d_6) $_2$-CH_3), 1.18−1.41 (m, 4H, -(CH_2)$_2$-CH_3), 3.01 (dd, $^2J_{AB}/^3J_{AX}$ = 16/1 Hz, 1H, -C*H*-H, H_A-5), 3.23 (dd, $^2J_{AB}/^3J_{BX}$ = 16/6 Hz, 1H, -CH-*H*, H_B-5), 4.69 (m, 1H, -CH-, H-6), 6.74 (dd, $J_{1/2}$ = 8/2 Hz, 1H, Ar-H), 6.80−6.90 (m, 3H, Ar-H), 7.83 (d, J = 8 Hz, 1H, Ar-H), 8.00 (d, J = 8 Hz, 1H, Ar-H), 9.40 (s, 1H, -OH), 10.05 (s, 1H, -OH), 10.26 (s, 1H, -CHO); IR (KBr) 3310, 1610 cm^{-1}. Anal. ($C_{20}H_{19}NO_3$) H, N; C: calcd, 74.75; found, 74.29.

6-Butyl-12-formyl-5,6-dihydro-3,9-dihydroxyindolo[2,1-*a*]isoquinoline (6c): prepared in 85% yield from 6-butyl-12-formyl-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (**5c**)[1] by a method similar to that decribed for **6a**; light-green crystals; mp 144−146 °C; ^{1}H NMR (DMSO-d_6)$_2$-CH_3), δ (t, J = 7 Hz, 3H, -CH_2-CH_3), 1.18−1.46 (m, 6H, -(CH_2)$_3$-CH_3), 3.01 (dd, $^2J_{AB}/^3J_{AX}$ = 16/1 Hz, 1H, -C*H*-H, H_A-5), 3.22 (dd, $^2J_{AB}/^3J_{BX}$ = 16/6 Hz, 1H, -CH-*H*, H_B-5), 4.68 (m, 1H, -CH-, H-6), 6.73 (dd, $J_{1/2}$ = 8/2 Hz, 1H, Ar-H), 6.80−6.89 (m, 3H, Ar-H), 7.83 (d, J = 8 Hz, 1H, Ar-H), 7.99 (d, J = 8 Hz, 1H, Ar-H), 9.40 (s, 1H, -OH), 10.04 (s, 1H, -OH), 10.25 (s, 1H, -CHO); IR (KBr) 3300, 1610 cm^{-1}. Anal. ($C_{21}H_{21}NO_3$) C, H, N.

6-Butyl-12-formyl-5,6-dihydro-3,10-dihydroxyindolo[2,1-*a*]isoquinoline (6d): prepared in 65% yield from 6-butyl-12-formyl-5,6-dihydro-3,10-dimethoxyindolo[2,1-*a*]isoquinoline (**5d**)[1] by a method similar to that described for **6a**; crystallization from Et_2O afforded beige crystals; dec >240 °C; ^{1}H NMR (DMSO-d_6) $_2$-CH_3), 1.15−1.45 (m, 6H, -(CH_2)$_3$-CH_3), 3.01 (dd, $^2J_{AB}/^3J_{AX}$ = 16/1 Hz, 1H, -C*H*-H, H_A-5), 3.24 (dd, $^2J_{AB}/^3J_{BX}$ = 16/6 Hz, 1H, -CH-*H*, H_B-5), 4.74 (m, 1H, -CH-, H-6), 6.73−6.86 (m, 3H, Ar-H), 7.39 (d, J = 9 Hz, 1H, Ar-H), 7.64 (d, J = 2 Hz, 1H, Ar-H), 7.84 (d, J = 8 Hz, 1H, Ar-H), 9.12 (s, 1H, -OH), 10.08 (s, 1H, -OH), 10.25 (s, 1H, -CHO); IR (KBr) 3265, 1615 cm^{-1}. Anal. ($C_{21}H_{21}NO_3$) C, H, N.

6-Butyl-12-formyl-5,6-dihydro-3,9,10-trihydroxyindolo[2,1-*a*]isoquinoline (6e): prepared in 70% yield from **5e** by a method similar to that described for **4**. Crystallization from $CHCl_3$ gave light-green crystals; mp 244−246 °C; ^{1}H-NMR ($CDCl_3$) δ 0.78 (t, ^3J = 7 Hz, 3H, -CH_2-CH_3), 1.15−1.41 (m, 6H, -(CH_2)$_3$-CH_3), 2.95, 3.01 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{AX}$ = 1 Hz, H_A-5), 3.16, 3.23 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{BX}$ = 6 Hz, 1H, H_B-5), 4.61 (m, 1H, H-6), 6.77−6.83 (m, 2H, H-2, H-4), 6.90 (s, 1H, H-8), 7.63 (s, 1H, H-11), 7.76 (d, ^3J = 8 Hz, 1H, H-1), 8.85 (s, 1H, OH), 8.95 (s, 1H, OH), 9.97 (1H, OH), 10.20 (s, 1H, -CH=O); IR (KBr) 1615 cm^{-1}. Anal. ($C_{21}H_{21}NO_4$) C, H, N.

12-[(*N*-Benzylimino)methyl]-6-butyl-5,6-dihydro-3,9-dihydroxyindolo[2,1-*a*]isoquinoline (7). After addition of ca. 50 mg of $CaSO_4$ and 0.5 mL of AcOH, the solution of **3c** (200 mg, 0.55 mmol) in a mixture of 3 mL of benzylamine and 7 mL of EtOH was stirred at room temperature under nitrogen atmosphere overnight. After filtration, the solvent and excess of amine were removed in vacuo. The residue was diluted with 50 mL of water and extracted with Et_2O. The combined organic layers were washed twice with water and dried (Na_2SO_4). After evaporation of the solvent a crystalline product was obtained which was recrystallized from Et_2O to give dark-red crystals (97%); dec >110 °C; ^{1}H NMR (acetone-d_6) $_2$)$_3$-CH_3), 1.22−1.55 (m, 6H, -(CH_2)$_3$-CH_3), 3.05 (dd, $^2J_{AB}/^3J_{AX}$ = 15/1 Hz, 1H, -C*H*-H, H_A-5), 3.27 (dd, $^2J_{AB}/^3J_{BX}$ = 15/6 Hz, 1H, -CH-*H*, H_B-5), 4.64 (m, 1H, -CH-, H-6), 4.85 (s, 2H, =N-CH_2-), 6.71 (dd, $J_{1/2}$ = 9/2 Hz, 1H, Ar-H), 6.80−6.91 (m, 3H, Ar-H), 7.19−7.46 (m, 5H, -C_6H_5), 7.71 (d, J = 8 Hz, 1H, Ar-H), 8.34 (d, J = 8 Hz, 1H, Ar-H), 8.40 (s, br, 2H, 2 × -OH), 9.02 (s, 1H, *H*-C=N-); IR (KBr) 3280, 1620 cm^{-1}; MS (EI) m/z 424 ($M^{\bullet+}$). Anal. ($C_{28}H_{28}N_2O_2$) C, H, N.

12-Acetyl-6-butyl-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (8). After addition of a catalytic amount of iodine, the mixture of 335 mg (1 mmol) of 6-butyl-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (**1c**) and 2 mL of Ac_2O was heated for 3 h at 150 °C. After cooling, ice water was added and the mixture extracted with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ solution, saturated $NaHSO_3$ solution, and water. After drying (Na_2SO_4), the solvent was removed and the residue chromatographed (SiO_2; CH_2Cl_2/EtOAc, 10:1). The solid obtained in 71% yield was recrystallized from Et_2O to give beige crystals: mp 115 °C; ^{1}H NMR ($CDCl_3$) δ 0.83 (t, $_2$-CH_3), 1.19−1.57 (m, 6H, -(CH_2)$_3$-CH_3), 2.64 (s, 3H, -CO-CH_3), 2.95 (dd, $^2J_{AB}/^3J_{AX}$ = 15/1 Hz, 1H, -C*H*-H, H_A-5), 3.34 (dd, $^2J_{AB}/^3J_{BX}$ = 15/6 Hz, 1H, -CH-*H*, H_B-5), 3.88 (s, 3H, -OCH_3), 3.90 (s, 3H, -OCH_3), 4.53 (m, 1H, -CH-, H-6), 6.80−6.92 (m, 4H, Ar-H), 7.92 (d, J = 9 Hz, 1H, Ar-H), 8.05 (d, J = 9 Hz, 1H, Ar-H); IR (KBr) 1630 cm^{-1}. Anal. ($C_{24}H_{27}NO_3$) C, H, N.

12-Acetyl-6-butyl-5,6-dihydro-3,9-dihydroxyindolo[2,1-*a*]isoquinoline (9): prepared in 67% yield from **8** by a method similar to that for **6a**; purification by chromatography (SiO_2; CH_2Cl_2/Et_2O, 1:1) gave light-green amorphous material; ^{1}H NMR (DMSO-d_6) $_2$-CH_3), 1.17−1.36 (m, 6H, -(CH_2)$_3$-CH_3), 2.51 (s, 3H, -CO-CH_3), 2.96 (dd, $^2J_{AB}/^3J_{AX}$ = 15/1 Hz, 1H, -C*H*-H, H_A-5), 3.19 (dd, $^2J_{AB}/^3J_{BX}$ = 15/6 Hz, 1H, -CH-*H*, H_B-5), 4.62 (m, 1H, -CH-, H-6), 6.67−6.84 (m, 4H, Ar-H), 7.72 (d, J = 9 Hz, 1H, Ar-H), 7.83 (d, J = 9 Hz, 1H, Ar-H), 9.29 (s, 1H, -OH), 9.88 (s, 1H, -OH); IR (KBr) 3305, 1625 cm^{-1}. Anal. ($C_{22}H_{23}NO_3$) C, H, N.

6-Butyl-5,6-dihydro-12-[(*N*-hydroxyimino)methyl]-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (10a). A solution of 400 mg (5.8 mmol) of hydroxylamine hydrochloride in 2 mL of water was added slowly to a solution of 200 mg (0.55 mmol) of 6-butyl-12-formyl-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (**5c**) in 4 mL of EtOH. After addition of ca. 50 mg of Na_2CO_3, the mixture was stirred overnight at room temperature. Then most of the solvent was removed in vacuo followed by addition of 30 mL of water. After extraction with

Et_2O, the organic layer was washed with water and dried (Na_2SO_4). After evaporation of the solvent the product (96%) was obtained as a crystalline solid. Recrystallization from MeOH/Et_2O (1:1) yielded colorless crystals: mp. 164 °C; ^{1}H NMR ($CDCl_3$) δ 0.83 (t, J = 7 Hz, 3H, -CH_2-CH_3), 1.18−1.62 (m, 6H, -(CH_2)$_3$-CH_3), 2.94 (dd, $^2J_{AB}/^3J_{AX}$ = 15/1 Hz, 1H, -CH-H, H_A-5), 3.29 (dd, $^2J_{AB}/^3J_{BX}$ = 15/6 Hz, 1H, -CH-H, H_B-5), 3.87 (s, 3H, -OCH_3), 3.90 (s, 3H, -OCH_3), 4.53 (m, 1H, -CH-, H-6), 6.79−6.96 (m, 5H, 4 × Ar-H, C=N-OH), 7.64 (d, J = 9 Hz, 1H, Ar-H), 8.07 (d, J = 9 Hz, 1H, Ar-H), 8.74 (s, 1H, H-C=N-OH). Anal. ($C_{23}H_{26}N_2O_3$) H, N; C: calcd, 72.99; found, 72.05.

6-Butyl-5,6-dihydro-12-[(*N*-hydroxyimino)methyl]-3,9,-10-trimethoxyindolo[2,1-*a*]isoquinoline (10b): prepared in 96% yield from **5e** by a method similar to that described for the preparation of **10a**; purified by column chromatography (SiO_2; CH_2Cl_2/EtOAc, 7:1) and recrystallized from MeOH/Et_2O (1:1); mp 143−145 °C; ^{1}H NMR ($CDCl_3$) δ 0.83 (t, ^3J = 7 Hz, 3H, -CH_2-CH_3), 1.19−1.68 (m, 6H, -(CH_2)$_3$-CH_3), 2.91, 2.97 (dd, $^2J_{AB}$ = 15 Hz, $^3J_{AX}$ = 1 Hz, 1H, H_A-5), 3.27, 3.34 (dd, $^2J_{AB}$ = 15 Hz, $^3J_{BX}$ = 6 Hz, 1H, H_B-5), 3.87 (s, 3H, -OCH_3), 3.97 (s, 3H, -OCH_3), 3.99 (s, 3H, -OCH_3), 4.49−4.56 (m, 1H, H-6), 6.80 (s, 1H, H-8), 6.84 (d, ^4J = 2 Hz, 1H, H-4), 6.88, 6.91 (dd, ^4J = 2 Hz, ^3J = 9 Hz, 1H, H-2), 7.40−7.50 (br, 1H, OH), 7.62 (d, ^3J = 9 Hz, 1H, H-1), 7.70 (s, 1H, H-11), 8.77 (s, 1H, H-C=N-OH). Anal. ($C_{24}H_{28}N_2O_4$) C, H, N.

6-Butyl-12-cyano-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (11a). Under nitrogen atmosphere, a solution of 1 mL of Ac_2O in 5 mL of dry pyridine was added dropwise to a mixture of 190 mg (0.5 mmol) of **7** and 100 mg of hydroxylamine hydrochloride in 5 mL of dry pyridine. The reaction mixture was heated to 100 °C for 3 h. After cooling, the mixture was poured into ice water and extracted with CH_2Cl_2. The combined organic layers were washed with 2 N HCl, saturated $NaHCO_3$ solution, and water and dried (Na_2SO_4). After evaporation of the solvent and removal of the excess of Ac_2O in vacuo, the residue was chromatographed (SiO_2; CH_2Cl_2/Et_2O, 50:1). The crystalline product (91%) was recrystallized from EtOH to give colorless crystals: mp 113−114 °C; ^{1}H NMR ($CDCl_3$) δ 0.8 (t, J = 7 Hz, 3H, -CH_2-CH_3), 1.16−1.61 (m, 6H, -(CH_2)$_3$-CH_3), 3.00 (dd, $^2J_{AB}/^3J_{AX}$ = 16/1 Hz, 1H, -CH-H, H_A-5), 3.37 (dd, $^2J_{AB}/^3J_{BX}$ = 16/6 Hz, 1H, -CH-H, H_B-5), 3.88 (s, 3H, -OCH_3), 3.89 (s, 3H, -OCH_3), 4.57 (m, 1H, -CH-, H-6), 6.81−6.83 (m, 2H, Ar-H), 6.89−6.96 (m, 2H, Ar-H), 7.60 (d, J = 9 Hz, 1H, Ar-H), 8.31 (d, J = 9 Hz, 1H, Ar-H); IR (KBr) 2210 cm^{-1}. Anal. ($C_{23}H_{24}N_2O_2$) C, H, N.

6-Butyl-12-cyano-5,6-dihydro-3,9,10-trimethoxyindolo[2,1-*a*]isoquinoline (11b): prepared in 77% yield from **10b** by a method similar to that described for **11a**. The product was purified by chromatography (SiO_2; CH_2Cl_2/Et_2O, 5:1) and recrystallization from EtOH; mp 145−147 °C; ^{1}H NMR ($CDCl_3$) δ 0.83 (t, ^3J = 7 Hz, 3H, -CH_2-CH_3), 1.15−1.61 (m, 6H, -(CH_2)$_3$-CH_3), 2.96, 3.02 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{AX}$ = 1 Hz, 1H, H_A-5), 3.35, 3.41 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{BX}$ = 6 Hz, 1H, H_B-5), 3.87 (s, 3H, -OCH_3), 3.96 (s, 3H, -OCH_3), 3.97 (s, 3H, -OCH_3), 4.52−4.60 (m, 1H, H-6), 6.80−6.82 (m, 2H, H-4, H-8), 6.90, 6.94 (dd, ^3J = 9 Hz, ^4J = 2 Hz, 1H, H-2), 7.14 (s, 1H, H-11), 8.27 (d, ^3J = 9 Hz, 1H, H-1); IR (KBr) 2199 cm^{-1}. Anal. ($C_{24}H_{26}N_2O_3$) C, H, N.

6-Butyl-12-cyano-5,6-dihydro-3,9-dihydroxyindolo[2,1-*a*]isoquinoline (12a): prepared in 75% yield from **11a** by a method similar to that described for **6a**; after chromatography (SiO_2; CH_2H_2/Et_2O, 2:1) beige crystals were obtained; mp 185−186 °C; ^{1}H NMR (DMSO-d_6) $_2$-CH_3), 1.15−1.43 (m, 6H, -(CH_2)$_3$-CH_3), 3.03 (dd, $^2J_{AB}/^3J_{AX}$ = 16/1 Hz, 1H, -CH-H, H_A-5), 3.27 (dd, $^2J_{AB}/^3J_{BX}$ = 16/6 Hz, 1H, -CH-H, H_B-5), 4.73 (m, 1H, -CH-, H-6), 6.76 (dd, $J_{1/2}$ = 9/2 Hz, 1H, Ar-H), 6.84−6.87 (m, 2H, Ar-H), 6.93 (d, J = 2 Hz, 1H, Ar-H), 7.37 (d, J = 9 Hz, 1H, Ar-H), 7.99 (d, J = 9 Hz, 1H, Ar-H), 9.50 (s, 1H, -OH), 10.06 (s, 1H, -OH); IR (KBr) 3290, 2210 cm^{-1}. Anal. ($C_{21}H_{20}N_2O_2$) H, N; C: calcd, 75.88; found, 75.40.

6-Butyl-12-cyano-5,6-dihydro-3,9,10-trihydroxyindolo[2,1-*a*]isoquinoline (12b): prepared in 78% yield from **11b** by a method similar to that described for **6e**; gray crystals; mp 200−202 °C; ^{1}H NMR ($CDCl_3$) δ 0.81 (t, ^3J = 7 Hz, 3H, -CH_2-CH_3), 1.21−1.58 (m, 6H, -(CH_2)$_3$-CH_3), 3.07, 3.13 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{AX}$ = 1 Hz, 1H, H_A-5), 3.33, 3.39 (dd, $^2J_{AB}$ = 16 Hz, $^3J_{BX}$ = 6 Hz, 1H, H_B-5), 4.70 (m, 1H, H-6), 6.90−7.04 (m, 4H, Ar-H), 7.90 (s, 1H, OH), 8.11 (d, ^3J = 9 Hz, 1H, H-1), 8.18 (s, 1H, OH), 8.89 (s, 1H, OH); IR (KBr) 2206 cm^{-1}. Anal. ($C_{21}H_{20}N_2O_3$) C, H, N.

6-Butyl-5,6-dihydro-3,9-dimethoxy-12-[(methylimino)-methyl]indolo[2,1-*a*]isoquinoline (13a). A 50−100-fold excess of aqueous methylamine was added to a solution of 200 mg (0.55 mmol) of 6-butyl-12-formyl-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (**2c**) in 10 mL of EtOH. After addition of 0.5 mL of acetic acid, the mixture was stirred overnight. After the removal of the solvent and the excess of amine in vacuo, 50 mL of water was added. The aqueous solution was extracted with Et_2O. The combined organic layers were washed twice with water and dried (Na_2SO_4). After the evaporation of the solvent the product (92%) was dried in vacuo. It was obtained as a brownish resin and required no further purification: ^{1}H NMR ($CDCl_3$) δ 0.82 (t, J = 7 Hz, 3H, -CH_2-CH_3), 1.19−1.59 (m, 6H, -(CH_2)$_3$-CH_3), 2.94 (dd, $^2J_{AB}/^3J_{AX}$ = 15/1 Hz, 1H, -CH-H, H_A-5), 3.30 (dd, $^2J_{AB}/^3J_{BX}$ = 15/6 Hz, 1H, -CH-H, H_B-5), 3.59 (d, J = 1 Hz, 3H, H-C=N-CH_3), 3.88 (s, 3H, -OCH_3), 3.89 (s, 3H, -OCH_3), 4.53 (m, 1H, -CH-, H-6), 6.78−6.94 (m, 4H, Ar-H), 7.69 (d, J = 9 Hz, 1H, Ar-H), 8.31 (d, J = 9 Hz, 1H, Ar-H), 8.83 (d, J = 1 Hz, 1H, H-C=N-CH_3).

12-[(Benzylimino)methyl]-6-butyl-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (13b). A 50−100-fold excess of benzylamine was added to a solution of 200 mg (0.55 mmol) of 6-butyl-12-formyl-5,6-dihydro-3,9-dimethoxyindolo-[2,1-*a*]isoquinoline (**2c**) in 10 mL of EtOH. After addition of ca. 100 mg of $CaSO_4$ and 0.5 mL of acetic acid, the mixture was stirred overnight. After the removal of the solvent and the excess of amine in vacuo, 50 mL of water was added. The aqueous solution was extracted with Et_2O. The combined organic layers were washed twice with water and dried (Na_2SO_4). After the evaporation of the solvent a solid product (80%) was obtained which crystallized from EtOH to give colorless crystals: mp 101 °C; ^{1}H NMR ($CDCl_3$) δ (t, J = 7 Hz, 3H, -(CH_2)$_3$-CH_3), 1.20−1.57 (m, 6H, -(CH_2)$_3$-CH_3), 2.95 (dd, $^2J_{AB}/^3J_{AX}$ = 15/1 Hz, 1H, -CH-H, H_A-5), 3.30 (dd, $^2J_{AB}/^3J_{BX}$ = 15/6 Hz, 1H, -CH-H, H_B-5), 3.87 (s, 3H, -OCH_3), 3.89 (s, 3H, -OCH_3), 4.54 (m, 1H, -CH-, H-6), 4.86 (d, $^2J_{AB}$ = 14 Hz, 1H, -CH-H-C_6H_5), 4.95 (d, $^2J_{AB}$ = 14 Hz, 1H, -CH-H-C_6H_5), 6.79 (d, J = 2 Hz, 1H, Ar-H), 6.83−6.92 (m, 3H, Ar-H), 7.22−7.46 (m, 5H, -C_6H_5), 7.67 (d, J = 9 Hz, 1H, Ar-H), 8.43 (d, J = 9 Hz, 1H, Ar-H), 8.94 (s, 1H, H-C=N-); IR (KBr) 1620 cm^{-1}.

6-Butyl-5,6-dihydro-3,9-dimethoxy-12-[(methylamino)-methyl]indolo[2,1-*a*]isoquinoline (14a). Sodium borohydride (50 mg) was added at 0 °C in small portions to a solution of 0.5 mmol of **10a** in 3 mL of MeOH. After stirring for 1 h at room temperature the mixture was heated under reflux for 45 min. After evaporation of the solvent in vacuo, CH_2Cl_2 and water (10 mL) were added and the layers separated. The aqueous solution was extracted with CH_2Cl_2, and the combined organic layers were washed with water and dried (Na_2SO_4). After evaporation of the solvent, the crude product was purified by chromatography over neutral alumina (activity III) with CH_2Cl_2 and an increasing proportion of Et_2O to give a light-brown resin (60%): ^{1}H NMR ($CDCl_3$) δ 0.82 (t, J = 7 Hz, 3H, -CH_2-CH_3), 1.13−1.69 (m, 7H, -(CH_2)$_3$-CH_3, NH), 2.59 (s, 3H, -NH-CH_3), 2.93 (dd, $^2J_{AB}/^3J_{AX}$ = 15/1 Hz, 1H, -CH-H, H_A-5), 3.28 (dd, $^2J_{AB}/^3J_{BX}$ = 15/6 Hz, 1H, -CH-H, H_B-5), 3.86 (s, 3H, -OCH_3), 3.89 (s, 3H, -OCH_3), 4.00 (d, $^2J_{AB}$ = 13 Hz, 1H, Ar-CH-H-NH-), 4.15 (d, $^2J_{AB}$ = 13 Hz, 1H, Ar-CH-H-NH-), 4.53 (m, 1H, -CH-, H-6), 6.73−6.84 (m, 3H, Ar-H), 6.91 (dd, $J_{1/2}$ = 9/3 Hz, 1H, Ar-H), 7.53 (d, J = 9 Hz, 1H, Ar-H), 7.88 (d, J = 9 Hz, 1H, Ar-H); MS (EI) m/z 378 ($M^{\bullet+}$). Anal. ($C_{24}H_{30}N_2O_2$) C, H, N.

12-[(Benzylamino)methyl]-6-butyl-5,6-dihydro-3,9-dimethoxyindolo[2,1-*a*]isoquinoline (14b): prepared from **10a** in 73% yield by a method similar to that described for **11a** to give a light-yellow resin; ^{1}H NMR ($CDCl_3$) δ 0.82 (t, J = 7 Hz, 3H, -CH_2-CH_3), 1.19−1.60 (m, 6H, -(CH_2)$_3$-CH_3), 1.68 (s, br, 1H, NH), 2.92 (dd, $^2J_{AB}/^3J_{AX}$ = 15/1 Hz, 1H, -CH-H, H_A-5), 3.26 (dd, $^2J_{AB}/^3J_{BX}$ = 15/6 Hz, 1H, -CH-H, H_B-5), 3.85 (s, 3H, -OCH_3), 3.88 (s, 3H, -OCH_3), 3.98 (d, J = 4 Hz, 2H, -NH-CH_2-C_6H_5), 4.01 (d, $^2J_{AB}$ = 13 Hz, 1H, Ar-CH-H-NH-), 4.19 (d, $^2J_{AB}$ = 13 Hz, 1H, Ar-CH-H-NH-), 4.51 (m, 1H, -CH-, H-6),

6.74−6.84 (m, 4H, Ar-H), 7.28−7.47 (m, 6H, -C_6H_5, Ar-H), 7.79 (d, J = 9 Hz, 1H, Ar-H). Anal. ($C_{30}H_{34}N_2O_2$) C, H, N.

Separation of the Enantiomers of 6b,c. The enantiomers of **6b,c** were separated semipreparatively by liquid chromatography on triacetylcellulose (20−30 μm)[30] with EtOH as eluent. The purity of the separated enantiomers was determined by HPLC (56 bar, 30 mL/min for **6b**; 26 bar, 15 mL/min for **6c**) using tris(phenylcarbamoyl)cellulose on SiO_2 as stationary phase, EtOH as eluent, and both UV spectroscopic (278 nm) and polarimetric (436 nm) detection. Capacity factors and optical purities: (+)-**6b**, $k'(+)_{436} = 0.04$, $P = 0.99$; (−)-**6b**, $k'(-)_{436} = 0.16$, $P = 0.94$; (+)-**6c**, $k'(+)_{436} = 0.01$, $P = 1.00$; (−)-**6c**, $k'(-)_{436} = 0.13$, $P = 0.97$.

Materials and Reagents for Bioassays. Drugs and biochemicals were obtained from Sigma (Deisenhofen, Germany). [^{3}H]Colchicine was purchased from New England Nuclear (Dreieich, Germany). Buffer solutions used: PEM−0.1 M PIPES-NaOH, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.6−6.7; PEMG−0.1 M PIPES-NaOH, 0.8 M monosodium L-glutamate, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.6−6.7; PBS−8.0 g/L NaCl, 0.2 g/L KCl, 1.0 g/L $Na_2HPO_4 \cdot H_2O$, 0.2 g/L KH_2PO_4. Scintillation liquid: Rotiszint eco plus (Roth, Karlsruhe, Germany).

Isolation and Purification of Calf Brain Tubulin. The cortex of one or two fresh calf brains in ice-cold PEM buffer (1 mL/g of tissue, + 16 mg of DTE/100 mL of buffer solution) was homogenized in portions. After centrifugation (90 min, 20000*g*) at 2−4 °C, the supernatant was carefully decanted. The concentrations of GTP and ATP were adjusted to 0.1 and 2.5 mM, respectively. After stirring gently at 37 °C for 30 min the solution was transferred to centrifugation tubes and carefully underlayered with a prewarmed (37 °C) sucrose solution (10% in PEM buffer solution containing 1 mM GTP, ca. 10% of the transferred volume). After centrifugation at 37 °C for 45 min (20000*g*) the pellets were weighed, suspended in ice-cold PEM buffer solution (3 mL/g), and homogenized in a Teflon-in-glas potter. After standing in ice for 30 min, the suspension was centrifuged at 2 °C for 30 min (40000*g*). The supernatant was removed and adjusted to 1 mM GTP. By incubation at 37 °C for 15 min tubulin was polymerized once again. After centrifugation at 37 °C for 30 min microtubuli were obtained as a shiny gel-like pellet. The yields ranged from 2 to 6 g/brain. Aliquots were frozen in liquid nitrogen and stored at −70 °C. Purity was checked by polyacrylamide gel electrophoresis.

Tubulin Polymerization Assay. The pellet of frozen microtubuli was warmed to 37 °C in a water bath. After addition of the 20-fold volume of ice-cold PEMG buffer, it was homogenized. Depolymerization was completed by keeping the mixture at 0 °C for 30 min followed by centrifugation at 2 °C (30 min, 30000*g*) to remove insoluble protein. Each reaction tube contained 0.46 mL of the supernatant and 20 μL of the DMSO solution of the test compound in varying concentrations. Reaction mixtures were preincubated at 37 °C for 15 min and chilled on ice followed by addition of 20 μL of a 25 mM GTP solution in PEMG buffer to each tube. Reaction mixtures were transferred to cuvettes of a UV spectrophotometer connected to two different temperature controllers. First, the temperature inside the cuvettes was held at 2 °C. The cuvette holder was then switched to the second temperature contoller at 37 °C, and the absorption was measured over a period of 20 min at 350 nm. Absorption at the start of the reaction was used as baseline. Two independent experiments were performed for the standard concentration (40 μM) and the three for determination of IC_{50} values. Each experiment had two control reaction mixtures; their mean value was defined as 100% and their turbidity readings were generally within 10% of each other.

Inhibition of the Binding of Colchicine to Tubulin. The tubulin solution was prepared as described above and diluted 1:10 with ice-cold PEM buffer. [^{3}H]Colchicine and test compounds were dissolved in DMSO. Each 0.1 mL reaction mixture contained 98 μL of the tubulin solution, 1 μM tritium-labeled colchicine, 10 μM inhibitor, and 2 mL of DMSO. Incubation was for 30 min at 37 °C. After the reaction mixture cooled on ice, 10 μL of a 11 mM GTP solution in PEM buffer was added, and the mixture was kept on ice. Each reaction mixture (0.1 mL) was filtered under reduced vacuum through a stack of four DEAE-cellulose paper filters and washed four times with 10 mL of PEM buffer. Radioactivity adsorbed on the filter representing tubulin-bound [^{3}H]colchicine was quantified in a liquid scintillation counter. Reaction mixtures without tubulin gave the background values.

Determination of Cytostatic Activity: 1. MDA-MB 231 Human Breast Cancer Cells. Hormone-independent human MDA-MB 231 breast cancer cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in McCoy-5a medium, supplemented with L-glutamine (73 mg/L), gentamycin sulfate (50 mg/L), $NaHCO_3$ (2.2 g/L), and 5% sterilized fetal calf serum (FCS). At the start of the experiment, the cell suspension was tranferred to 96-well microplates (100 μL/well). After the cells grew for 2−3 days in a humidified incubator with 5% CO_2 at 37 °C, medium was replaced by one containing the test compounds (200 μL/well). Control wells (16/plate) contained 0.1% DMF that was used for the preparation of stock solutions. Initial cell density was determined by addition of glutaric dialdehyde (1% in PBS; 100 μL/well) instead of test compound. After incubation for about 4 days, medium was removed and 100 μL of glutaric dialdehyde in PBS (1%) was added for fixation. After 15 min, the solution of aldehyde was decanted. Cells were stained by treating them for 25 min with 100 μL of an aqueous solution of crystal violet (0.02%). After decanting, cells were washed several times with water to remove adherent dye. After addition of 100 μL of EtOH (70%), plates were gently shaken for 2 h. Optical density of each well was measured in a microplate autoreader EL 309 (Bio-tek) at 578 nm.

2. MCF-7 Human Breast Cancer Cells. A similar procedure to that described for MDA-MB 231 cells was applied with alterations: Cells were grown in EMEM supplemented with sodium pyruvate (110 mg/L), gentamycin sulfate (50 mg/L), $NaHCO_3$ (2.2 g/L), and 10% FCS. Medium that contained test compounds was supplemented with dextran-charcoal (DCC)-treated FCS to avoid interference with steroidal hormones in the serum. Incubation with inhibitor lasted ca. 8 days.

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