Síntesis y evaluación biológica de agentes antitubulínicos relacionados con colchicina y combretastatina

Synthesis and biological evaluation of antitubulin agents related to colchicine and combretastatin

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RESUMEN

La tubulina representa un blanco promisorio para las drogas antitumorales debido a su importancia, tanto en el crecimiento de las células tumorales, como de las células endoteliales responsables de la angiogénesis en tumores sólidos. Las combretastatinas, aisladas de la planta *Combretum caffrum*, inhiben la polimerización de los microtúbulos. Existe un gran interés en sintetizar análogos basados en una de ellas, la combretastatina A-4 (CA-4), preservando algunos detalles de su estructura, pero al mismo tiempo mejorando su solubilidad y actividad antitumoral. En este estudio sintetizamos cuatro compuestos relacionados con la combretastatina y evaluamos su efecto sobre la viabilidad, migración y citoesqueleto de líneas tumorales y endoteliales. El compuesto **5a** (1-(3,4,5-trimetoxifenil)-6,7-metilendioxi-3,4-dihidroisoquinolina)) inhibió tanto la proliferación como la migración en tres líneas celulares evaluadas. Además, se observó por microscopía confocal, una despolimerización y desintegración de los microtúbulos en las células a concentraciones no-citotóxicas del compuesto **5a** (1 µg/ml).

Palabras clave: droga antitubulina, cáncer, combretastatina A-4, citotoxicidad, migración.

ABSTRACT

Tubulin represents a promising target for new antitumour drugs as it is important both in cancer cells growth, as well as in the endothelial cells responsible for angiogenesis in solid tumors. One of such group of drugs is the combrestastatins, isolated from the plant *Combretum caffrum*, which inhibit microtubule polymerization. There is great interest in synthesizing analogues based on one of them, combretastatin A-4 (CA-4), preserving some structural details but improving its solubility and antitumor activity. In this study we synthesized four compounds related to combretastatin and evaluated their effect on the viability, migration and the cytoskeleton of tumor and endothelial cell lines. Compound **5a** (1-(3,4,5-trimethoxyphenyl)-6,7-methylenedioxy-3,4-dihydroisoquinoline)) inhibited both cell proliferation and migration. Also, as determined by confocal microscopy, this compound induced microtubule depolymerization and disruption in the three cell lines tested, at a non-cytotoxic concentration (1 µg/ml).

Keywords: antitubulin drug, cancer, combetastatin A-4, cytotoxicity, migration

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Introduction

Among the many inhibitors of cell growth and mitosis, some exert their effect by interfering with the polymerization and depolymerization of tubulin, interrupting the formation of microtubules that constitute the mitotic spindle, causing mitotic arrest in the M phase of the cell cycle and finally resulting in cell death by apoptosis. Tumor cells are more sensitive to such agents than normal cells due to their higher rate of cell division.

Some of these compounds with diverse molecular structures are 2-methoxyestradiol (Cushman et al., 2002), curacin A (Verdier-Pinard et al., 1998), some 2-phenyl-4-quinolones (Li et al., 1994), podophyllotoxin (Cortese et al., 1977), steganacin (Wang et al., 2008) and combretastatin A-4 (CA-4) (Pettit et al., 1995), which is one of the more potent inhibitors of tubulin polymerization known to date and which interacts with tubulin in the same way as colchicine, at the «colchicine-binding site». Two cell types are important targets when these antitubulin drugs are employed as anticancer agents, the tumor cell itself and the host endothelial cell of the vasculature which is essential for the tumor's survival. Drugs such as the vinca alkaloids act directly on the former whereas angiogenesis inhibitors also act on the endothelial cell depriving the tumor of its blood supply. Several compounds share the two effects, among them, colchicine, podophyllotoxin and CA-4, but except for CA-4, this effect is observed close to the maximum tolerated dose (Tron et al., 2006).

The potent antivascular and antitumour activities of CA-4 have stimulated research into the design and synthesis of a variety of analogues and derivatives. Most of these syntheses have tended to freeze the *cis* configuration of the double bond that links the two aromatic rings in CA-4, incorporating the double bond into a five or six member ring (Pirali et al., 2006; Simoni et al., 2005; Tron et al., 2005).

Our group is searching for analogues of antitubulin drugs with high activity, synthesizing compounds with simple molecular structures but maintaining the structural characteristics common to colchicine, steganacin, podophyllotoxin and CA-4, specifically the trimethoxybenzene ring and a second aromatic ring with two adjacent oxygenated substituent's. Here we present results showing the biological activity of two 3,4-dihydroisoquinolines and their corresponding phenethyl-3,4,5-trimethoxybenzamide precursors.

Materials and methods

Chemistry

The melting points were measured in an Electrothermal apparatus and were not corrected. The infrared spectra were performed on thin films, as a $CHCl_3$ solution or in KBr pellet, depending on the compound and measured in a Perkin Elmer 1310 spectrophotometer. The wave numbers were expressed in cm^{-1}.

The ^1H and ^{13}C-NMR spectra were determined in 270 MHz JEOL ECLIPSE and 300 MHz BRUKER AVANCE spectrometers. The solvent used is specified individually for each compound. The chemical shifts (δ) are expressed in ppm at lower field of TMS signal.

General proceeding for preparation of β-E- nitrostyrenes, 1

Substituted benzaldehyde (2.56 mmoles) was reacted with 3 mL (55.43 mmoles) of nitromethane, 2.77 mmoles of methylamine chlorhydrate and 1.83 mmoles of sodium acetate. The mixture was stirred at room temperature for 20 hours. Water was then added and the product extracted with CH_2Cl_2 then dried over Na_2SO_4, leaving a yellow solid.

3,4-Methylenedioxy-β-E-nitrostyrene, **1a**

Yield 99%. mp 159-161°C. IR ($CHCl_3$) ν_{max}: 3020, 1610, 1330, 1250 cm^{-1}

^1H-NMR ($CDCl_3$) δ : 7.90 (d, 1H, H-α, J= 13.52 Hz), 7.45 (d, 1H, H-β, J = 13.53 Hz), 7.05 (d, 1H, H-6, J = 7.91 Hz), 6.98 (d, 1H, H-2, J = 1.67 Hz), 6.85 (d, 1H, H-5, J = 7.91 Hz), 6.04 (s, 2H, $-CH_2-$) . ^{13}C-NMR ($CDCl_3$) δ : 151.45 (C-3), 148.82 (C-4), 139.19 (C-α), 135.43 (C-β), 126.73 (C-6), 124.24 (C-1), 109.14 (C-2), 107.05 (C-5), 102.15 (CH_2).

3,4-Dimethoxy-β-E-nitrostyrene, **1b**

Yield 99%. mp 136-138°C. IR ($CHCl_3$) ν_{max}: 3000, 1620, 1500, 1320, 1270 cm^{-1}. ^1H-NMR ($CDCl_3$) δ : 7.94 (d, 1H, H-α, J = 13.33 Hz), 7.51 (d, 1H, H-β, J = 13.60 Hz), 7.15 (dd, 1H, H-6, J = 8.15, 1.97 Hz), 6.98 (d, 1H, H-2, J = 1.97 Hz), 6.89 (d, 1H, H-5, J = 8.42), 3.91 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3). ^{13}C-NMR ($CDCl_3$) δ : 152.86 (C-3), 149.60 (C-4),; 139.43 (C-α), 135.22 (C-β), 124.73 (C-6), 122.86 (C-1), 111.40 (C-2), 110.29 (C-5), 56.16 (OCH_3), 56.09 (OCH_3).

General proceeding for preparation of nitrophenethyls, 2

Nitrostyrene (2.66 mmoles) was dissolved in 15 mL of ethanol, then 7.76 mmoles of dimethylsulphoxide (DMSO) were added and the solution maintained at -5°C with stirring. Sodium borohydride (5.34 mmoles) was then added slowly over a period of 1 hour. The mixture was treated with dilute HCl, the ethanol

was evaporated and the product extracted with CH_2Cl_2 then dried over Na_2SO_4. After purification by column chromatography, using CH_2Cl_2-C_6H_{14} mixtures as eluent, a dense oil was obtained.

3,4-Methylenedioxynitrophenethyl**, 2a**

Yield 87%. IR ($CHCl_3$) ν_{max} : 2880, 1550, 1240, 1030 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ : 6.69 (m, 3H, H-2,5,6), 5.92 (s, 2H, -CH_2-), 4.54 (t, 2H, H-α, J = 7.18 Hz), 3.20 (t, 2H, H-β, J = 7.18 Hz). ^{13}C-NMR ($CDCl_3$) δ: 148.10 (C-3), 146.99 (C-4), 129.28 (C-1), 121.79 (C-6), 108.96 (C-2), 108.70 (C-5), 101.20 (-CH_2-), 76.58 (C-α), 33.28 (C-β).

3,4-Dimethoxynitrophenethyl**, 2b**

Yield 50%. IR ($CHCl_3$) ν_{max} : 3000, 1540, 1250, 1130 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ : 6.73 (m, 3H, H-2,5,6), 4.56 (t, 2H, H-α, J = 7.26 Hz), 3.84 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.23 (t, 2H, H-β, J = 7.26 Hz). ^{13}C-NMR ($CDCl_3$) δ : 149.27 (C-3), 148.42 (C-4), 128.17 (C-1), 120.70 (C-6), 111.60 (C-2), 111.42 (C-5), 55.96 (OCH_3), 55.57 (OCH_3), 76.57 (C-α), 33.20 (C-β).

General proceeding for preparation of phenethylamines, 3

Nitrophenethyl (3.19 mmoles) and $NiCl_2.6H_20$ (6.33 mmoles) were dissolved in 15 mL of ethanol. Sodium borohydride (16.36 mmoles) was then added slowly over a period of 1 hour. After removal of the ethanol by evaporation, the mixture was dissolved in CH_2Cl_2 and extracted with concentrated HCl. After basification of the aqueous phase and CH_2Cl_2 extraction, a yellow oil was obtained.

3,4-Methylenedioxyphenethylamine**, 3a**

Yield 67%. IR ($CHCl_3$) ν_{max}: 3360, 2920, 1600, 1480, 1240 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.65 (m, 3H, H-2,5,6), 5.88 (s, 2H, -CH_2-), 2.87 (t, 2H, H-a, J= 6.66 Hz), 2.62 (t, 2H, H-b, J= 6.66 Hz), 1.63 (s, 2H, NH_2). ^{13}C-NMR ($CDCl_3$) δ: 147.71 (C-3), 145.96 (C-4), 133.57 (C-1), 121.73 (C-6), 109.18 (C-2), 108.27 (C-5), 100.86 (-CH_2-), 43.50 (C-α); 39.69 (C-β).

3,4-Dimethoxyphenethylamine**, 3b**

Yield 68%. IR ($CHCl_3$) ν_{max}: 3360, 2920, 1580, 1500, 1150 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.71 (m, 3H, H-2,5,6), 3.83 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 2.91 (t, 2H, H-a, J= 6,93 Hz), 2.66 (t, 2H, H-b, J= 6,93), 1.99 (brs, 2H, NH_2). ^{13}C-NMR ($CDCl_3$) δ: 149.00 (C-3), 147.55 (C-4), 132.28 (C-1), 120.79 (C-6), 112.13 (C-2), 111.40 (C-5), 55.98 (OCH_3), 55.90 (OCH_3), 43.51 (C-α), 39.34 (C-β).

General proceeding for preparation of phenethylbenzamides, 4

Phenethylamine (3.18 mmoles) and triethylamine (3.69 mmoles) were dissolved in dry chloroform and stirred at -5°C. 3,4,5-trimethoxybenzoyl chloride (3.29 mmoles), dissolved in a minimum quantity of chloroform, was then slowly added and the reaction maintained at room temperatture for 16 hours. The reation mixture was extracted with 10% $NaHCO_3$ in water then dried over Na_2SO_4. White crystals were obtained on recrystallization from ethanol.

N-(3,4-methylenedioxyphenethyl)-3,4,5-trimethoxybenzamide**, 4a**

Yield 97%. mp 135-136°C. IR ($CHCl_3$) ν_{max}: 3290, 2920, 1630, 1580, 1120 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.90 (s, 2H, H-2',6'), 6.67 (m, 3H, H-2,5,6), 6.28 (brs, 1H, NH), 5,88 (s, 2H, -CH_2-), 3.82 (s, 3H, OCH_3), 3.81 (s, 6H, OCH_3), 3.59 (c, 2H, H-a, J= 6,93 Hz), 2.79 (t, 2H, H-b, J= 6,93 Hz). ^{13}C-NMR ($CDCl_3$) δ: 167.29 (C=O), 153.19 (3',5'), 147.94 (C-3), 146.31 (C-4), 140.83 (C-4'), 132.71 (C-1'), 130.18 (C-1) 121.76 (C-6), 109.19 (C-2), 108.40 (C-5), 104.29 (C-2',6'), 100.99 (-CH_2-), 60.93 (4'-OCH_3), 56.26 (3',5'-OCH_3), 41.45 (C-α), 35.46 (C-β).

N-(3,4-Dimethoxyphenethyl)-3,4,5-trimethoxybenzamide**, 4b**

Yield 95%. mp 133-134°C. IR ($CHCl_3$) ν_{max}: 3440, 3000, 1650, 1580, 1220, 1130 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.89 (s, 2H, H-2',6'), 6.73 (m, 3H, H-2,5,6), 6.26 (brs, 1H, NH), 3.80 (s, 15H, OCH_3), 3.62 (c, 2H, H-a, J= 6,93 Hz), 2.82 (t, 2H, H-b, J= 6,93 Hz). ^{13}C-NMR ($CDCl_3$) δ: 167.26 (C=O), 153.19 (C-3',5'), 149.15 (C-3), 147.81 (C-4), 140.87 (C-4'), 131.50 (C-1'), 130.17 (C-1), 120.79 (C-6) 112.13 (C-2), 111.43 (C-5), 104.31 (C-2',6'), 60.93 (4'-OCH_3), 56.27 (3',5'-OCH_3), 55.97 (OCH_3), 55.91 (OCH_3), 41.45 (C-α), 35.25 (C-β).

General proceeding for preparation of 3,4-Dihydroisoquinolines, 5

The corresponding amide (0.57 mmoles) was mixed with an excess of phosphorus oxychloride (40 mmoles), heated to reflux for 2 hours then cooled on ice to room temperature. The aqueous layer was basified and extracted with dichloromethane. Preparative thin layer chromatography yielded a light yellow solid.

1-(3,4,5-Trimethoxyphenyl)-6,7-methylenedioxy-3,4-dihydroisoquinoline, **5a**

Yield 87%. mp 155°C. IR($CHCl_3$) ν_{max}: 2940, 1570, 1480, 1370, 1220, 1130 cm^{-1}. ^{1}H-NMR ($CDCl_3$)

δ: 6.78 (s, 1H, H-8), 6.77 (s, 2H, H-2',6'), 6.73 (s, 1H, H-5), 5.95 (s, 2H, -CH_2-), 3.86 (s, 3H, 4'-OCH_3), 3.84 (s, 6H, 3',5'-OCH_3), 3.73 (t, 2H, , H-3, J= 7,58 Hz), 2.68 (t, 2H, H-4, J= 7,58 Hz). ^{13}C-NMR ($CDCl_3$) δ: 166.64 (C-1), 153.02 (C-3', 5'), 149.35 (C-6), 146.21 (C-7), 141.00 (C-4'), 134.48 (C-4a,8a), 122.51 (C-1'), 108.70 (C-8), 108.00 (C-5), 106.16 (C-2', 6'), 101.42 (-CH_2-), 60.98 (4'-OCH_3), 56.31 (3',5'-OCH_3), 47.37 (C-3), 26.65 (C-4).

1-(3,4,5-Trimethoxyphenyl)-6,7-dimethoxy-3,4-dihydroisoquinoline, **5b**

Yield 86%. mp 158°C. IR ($CHCl_3$) ν_{max}: 2920, 1550, 1350, 1120 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.85 (s, 1H, H-8), 6.83 (s, 2H, H-2,6'), 6.77 (s, 1H, H-5), 3.93 (s, 3H, 6-OCH_3), 3.87 (s, 3H, 4'-OCH_3), 3.84 (s, 6H, 3',5'-OCH_3), 3.78 (t, 2H, H-3, J= 7,66 Hz), 3.73 (s, 3H, 7-OCH_3), 2.71 (t, 2H, H-4, J= 7,66 Hz). ^{13}C-NMR ($CDCl_3$) δ: 166.65 (C-1), 153.02 (C-3',5'), 151.17 (C-6), 147.13 (C-7), 139.18 (C-4'), 134.39 (C-4a), 132.99 (C-8a), 121.12 (C-1'), 111.68 (C-8), 110.35 (C-5), 106.19 (C-2',6'), 61.00 (4'-OCH_3), 56.27 (6,7,3',5'-OCH_3), 47.41 (C-3), 26.09 (C-4).

Cell lines

Two tumor and one endothelial cell lines were used: B16/BL6 (mouse melanoma), CT26 (mouse mammary tumor) and LSEC (mouse liver synovial endothelium – kindly provided by Dr. José Cardier). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine, (Gibco, BRL, USA) and penicillin (100 Units/ml) – streptomycin (100 µg/ml).

Growth inhibition and cytotoxicity

The sulforhodamine B (SRB) assay was used to evaluate the effect of the compounds on the growth and viability of the cell lines (Skehan et al., 1990). Each drug was assayed in triplicate at 6 concentrations up to 100 µg/ml. The concentrations inducing 50% growth inhibition (GI_{50}), total growth inhibition (TGI) and 50% cytotoxicity (LC_{50}) were calculated by linear interpolation from the observed data points.

Wound assay

Inhibition of cell migration was measured as described previously (Liang et al., 2007). Cell lines were grown in 96-well plates at 10^5 cells/well and after 24 h, a line was scratched through the cell monolayer with a pipette tip. Wounded monolayer's were washed once with medium then incubated with the compound. Microphotographs were taken at 0 , 21 and 36 h. The inhibition of migration was calculated from the width of the wound in triplicate wells at the different time points in the presence of the drug compare to the control wells.

Confocal microscopy

The effect of exposure to compound **5a** on the cellular cytoskeleton was assessed by confocal microscopy. Cell were allowed to adhere for 24 h on chamber slides (Lab-Tek, Fisher Scientific, USA), then incubated for a further 24 h in the presence of the compound at 0, 1 and 3 µg/ml. The cells were fixed in 4% paraformaldehyde, permeabilized with 1% Triton X-100, then incubated with mouse monoclonal antitubulin antibody (1:500, Sigma-Aldrich, USA) for 1 h at 25°C, followed by a secondary anti mouse FITC–conjugated antibody (1:200, Sigma-Aldrich) for another hour. After washing, the samples were dried, covered with mounting solution (Mowiol 4-88, Calbiochem, USA) then examined at a magnification of x1000 using an inverted Nikon Eclipse TE2000U microscope equipped with a confocal Nikon C1 system with an argon laser at 488 nm excitation. Images were acquired with the Nikon EZ-C1 software.

Statistics

The results were expressed as the mean value ± SD (standard deviation). The unpaired Student's t test with the Welch correction was used to assess the statistical significance of the differences.

Results

Chemistry

The resulting compounds, shown in Figure 1, were:

4a : N-(3,4-methylenedioxyphenethyl)-3,4,5-`trimethoxybenzamide

4b : N-(3,4-dimethoxyphenethyl)-3,4,5-trimethoxybenzamide

5a : 1-(3,4,5-trimethoxyphenyl)-6,7-methylenedioxy-3,4-dihydroisoquinoline

5b : 1-(3,4,5-trimethoxyphenyl)-6,7-dimethoxy-3,4-dihydroisoquinoline

These compounds have been obtained previously in synthetic studies of some orchidaceae alkaloids (Leander and Lüning, 1969) and derivatives of podophylotoxin and related compounds (Gensler and Samour, 1950; Reeve and Eareckson, 1950). We obtained them in good yields and then were tested to determine their possible biological activities.

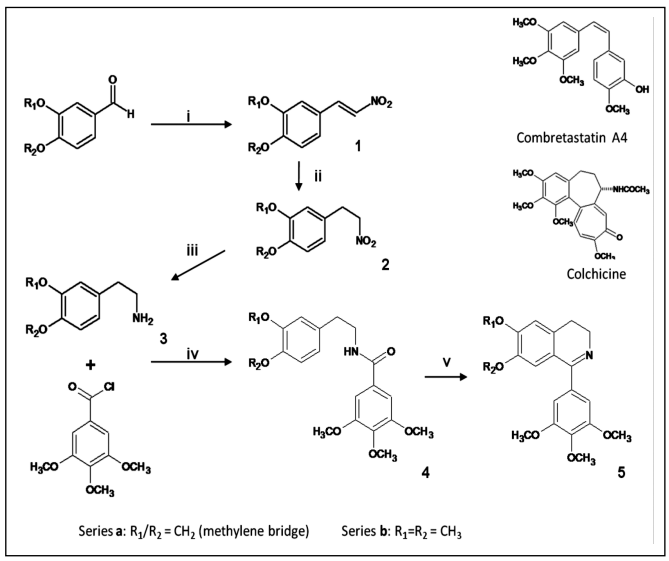

Figure 1. Scheme of synthesis. Reagents: i) CH_3NO_2, AcONa, $CH_3NH^+Cl^-$ ii) $NaBH_4$/DMSO iii) $NiCl_2.6H_2O/NaBH_4$ iv) $N(CH_2CH_3)$/ $CHCl_3$ v) $POCl_3$

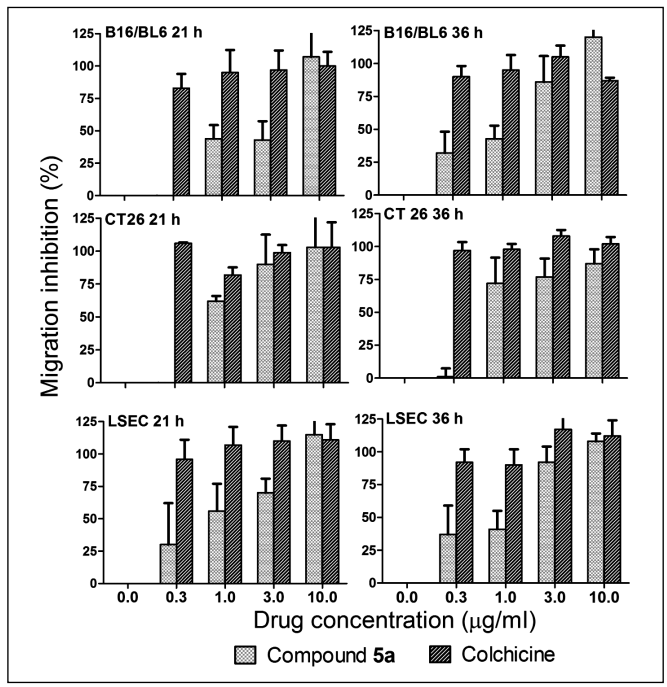

Figure 2. Effect of compound number 5a on the migration of three cell lines (B16/BL6, CT26 and LSEC-1). The wound assay was used to determine the effect of compound 5a on cell migration. Images of the wound gaps were acquired at 21 and 36 h to determine the degree of closure. The means and standard deviations of triplicate wells from one of two experiments are shown. *P<0.05, **P<0.01, compared to the untreated control group.

GROWTH INHIBITION AND CYTOTOXICITY

All the compounds were cytostatic at concentrations below 100 µg/ml with no cytotoxic activity at the concentrations tested (Table I). However, the effect of compound **5a** on cell division was markedly greater than the other three with values for GI_{50} less than 3 µg/ml for all 3 cell lines.

WOUND ASSAY

Preliminary assays indicated that compounds **4a**, **4b** and **5b** only inhibited cell migration at concentrations close to 100 µg/ml (results not shown), whereas **5a** was inhibitory at much lower concentrations. The results for this compound, which are fairly similar at 21 and 36 h, are shown in Figure 2. After a 36 h incubation, **5a** inhibited B16/BL6 and LSEC cells migration by more than 30%, at doses as low as 0.3 µg/ml. Migration of all 3 cell types was almost totally inhibited at 3 µg/ml.

CYTOSKELETON

The positive results shown by **5a** on cell proliferation and migration led us to determine the effect of this drug on the cytoskeleton of cells in culture. After 24 h in the presence of low concentrations of this compound, confocal microscopy with an antitubulin antibody revealed a clear disruption of the cytoskeleton in all three cell lines (Figure 3). At 1 µg/ml, cell rounding and disorganization of the microtubules was evident, whereas at 3 µg/ml, cell rounding was more pronounced and only shorter tubule segments could be seen in all 3 cell lines. There was a general reduc-

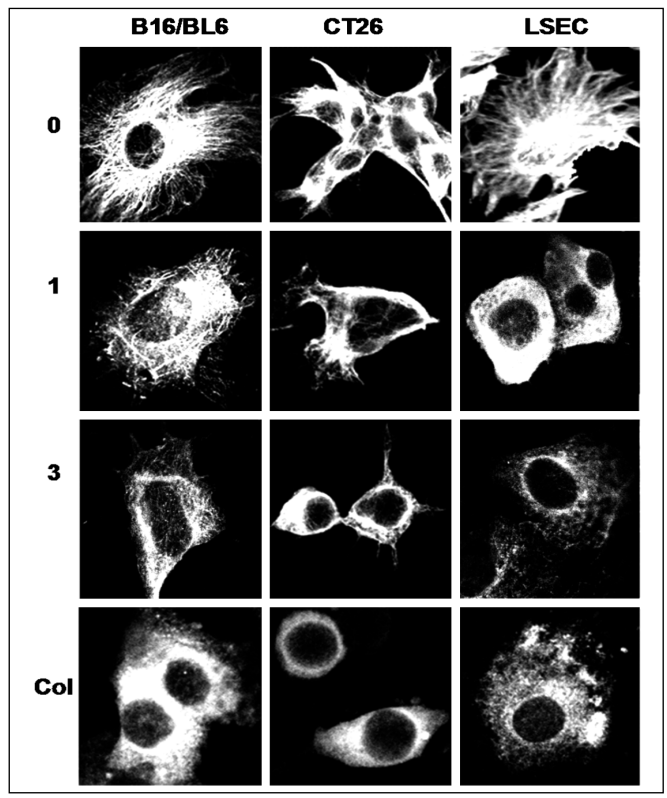

Figure 3. Sensitivity of the cytoskeleton of the three cell lines: Bl6/BL6, CT26 and LSEC to compound 5a. Proliferating cells were treated with the indicated concentrations of 5a (left hand side - µg/ml) for 24 h then fixed and immunostained with a fluorescently conjugated antibody to α-tubulin. Colchicine (1 µg/ml) was used as the positive control. Confocal microscopy - 100x.

Table I

Growth inhibitory effects of the four compounds on B16/BL6, CT26 and LSEC cells

	B16/BL6.			CT26.			LSEC.		
µg/ml	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
4[a]	66.3	>100	>100	61.0	>100	>100	24.0	>100	>100
4b	68.9	>100	>100	96.9	>100	>100	26.4	>100	>100
5[a]	2.7	74.2	>100	1.0	89.2	>100	2.9	93.7	>100
5b	20.4	68.2	96.4	36.1	91.6	>100	20.1	>100	>100
Colchicine	0.1	>100	>100	0.2	>100	>100	0.1	0.7	>100

GI50 – concentration causing 50% inhibition of cell growth. TGI – concentration for total growth inhibition, LC50 – concentration causing 50% cytotoxicity. The data are representative of at least 2 experiments performed in triplicate.

tion in the microtubule polymer mass. The endothelial cell line appeared to be somewhat more sensitive to the drug than the two tumor lines. The known powerful effect of the control drug colchicines, at 1 µg/ml was evident on all three cell lines.

DISCUSSION

Since 1971, when Folkman first proposed the tumor vasculature as a potential target for therapy (Folkman, 1971), many antiangiogenic drugs have been investigated. The effect of CA-4, and its more soluble phosphate form (CA-4P), against the proliferation and migration of malignant cells has been studied for many years (Nam, 2003). As the aim of our research was to obtain compounds with structural characteristics similar to known inhibitors of tubulin polimerization, such as CA-4, they would necessarily need to be small molecules. Our goal was to place the structural details of CA-4 and other tubulin inhibitors on common structures in natural products such as the nitrogen heterocycles, quinoline or isoquinoline. These are simple structures and a variety of syntheses are known for them.

In the particular case of the isoquinoline derivatives as those prepared in the present article, the two best known reactions for their synthesis are the Bishler-Napieralski (Chrzanowska and Rozwadowska, 2004) and the Pictet-Spengler (Whaley and Govindachari, 1951). In either case, it is necessary to obtain the phenethylamines with the desired substituents on the phenyl ring. To do this, the suitably substituted benzaldehydes are treated with nitromethane, using an amine salt with mineral or organic acid as catalyst (Henry reaction), obtaining the corresponding nitrostyrenes. The yields of these reactions are affected by the substituents on the ring, but they are usually good. Although the reduction of nitrostyrenes to phenethylamines is usually carried out with lithium aluminium hydride, for this synthesis we decided to try two consecutive reactions with cheaper reagents and a quite good overall yield (see Results). The Bishler-Napieralski reaction was used to obtain the 3,4-dihydroisoquinolines because of the greater stability of intermediate amides as compared to the imines, which are intermediate in the Pictet-Spengler reaction. Since the amides obtained also show structural characteristics with possible biological activity, they were also tested in the assays.

All four compounds synthesized were cytostatic but not cytotoxic. Anti-angiogenic drugs mostly show cytostatic activity and are thought to act by stabilizing tumors and thus preventing metastasis (Eckhardt, 1999). Although all four compounds inhibited cell proliferation in a dose-dependent manner, **5a** was by far the more active, followed by **5b**. This difference may be due to steric hindrance of the methoxy group in **5b** preventing binding to the colchicine-binding site. Although much effort has been dedicate to the preparation of more water soluble drugs, such as CA-4P, for clinical oral use, the ability of a drug to pass the cell membrane must be considered as well.

It is known that colchicine inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules. Availability of tubulin is essential to mitosis, and therefore colchicine effectively functions as a «mitotic poison» or spindle poison inhibiting microtubule polymerization and interrupting the cell cycle in mitosis (Jordan, 2002). Colchicine, combretastatin and our compounds have common structural characteristics such as a trimethoxybenzene ring and an aromatic ring with two adjacent oxygen groups.

Despite the slower rate of cell division of the endothelial cell line, the three biological assays showed

a tendency towards a greater susceptibility of these cells to **5a**. These results agree with the previous observations in which is demonstrated that endothelial cells are especially vulnerable to tubulin-binding compounds (Dark et al., 1997; Kanthou et al., 2004; Tozer et al., 1999). Kanthou et al. analyzed the mechanism by which combretastatin induces the death of proliferating endothelial cells, observing that induction of apoptosis in endothelial cells by this drug is associated with prolonged mitotic arrest.

Further studies are required to determine the exact mode of action of compound **5a** on tubulin and *in vivo* studies to establish its efficacy in a mouse tumor model, in order to propose compound **5a** as a possible antitumor drug.

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