European Journal of Medicinal Chemistry 48 (2012) 108–113

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and biological evaluation of novel indolocarbazoles with anti-angiogenic activity

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ARTICLE INFO

Article history:
Received 17 October 2011
Received in revised form
22 November 2011
Accepted 28 November 2011
Available online 7 December 2011

Keywords:
Angiogenesis
Indolocarbazole
HUVEC
Staurosporine analogs

ABSTRACT

A novel series of indolocarbazoles were synthesized and their antiproliferative activity against HUVEC, LoVo, DLD-1 and ST-486 cell lines, was investigated. Those staurosporine analogs in which a substituted dimethylaminoalkoxy chain was attached to the indolic nitrogen showed interesting activity and selectivity with respect to HUVEC proliferation. The effect on capillary tube formation in 3-dimensional matrigel matrix was studied using the most active compounds. Evaluation of their *in vivo* anti-angiogenic activity in a murine Lewis lung cancer model was also analyzed.

1. Introduction

Angiogenesis is a process of development and growth of new capillary blood vessels involved in physiological events such as embryogenesis, organ development and wound healing [1]. However, angiogenesis can become pathological, and contribute to the development of certain diseased states including retinopathy, rheumatoid arthritis, atherosclerosis, psoriasis, hemangioma, tumor development and the formation of metastases [2,3]. As cancer progression, invasiveness and metastases are angiogenesis dependant, control of this process is one of the therapeutic approaches used in oncology [4].

Vascular endothelial growth factor (VEGF) is an important modulator of angiogenesis which regulates vascular permeability, endothelial cell growth, and migration [5]. However angiogenesis is not just regulated by soluble growth factors, but also by cellular interactions with extracellular matrix which plays an important role in cell adhesion, differentiation, and proliferation [6]. Capillary tube formation had been used as a model to test *in vitro* angiogenesis, as endothelial tube network formed *in vitro* has many similarities with capillary vessels formed *in vivo* [7].

After *in vitro* assays, it appeared necessary to investigate the potential effect of an angiogenesis inhibitor with an *in vivo* assay, to ensure that the results seen *in vitro* translate to the *in vivo* state [8]. The classical model of the Lewis lung carcinoma has been extensively used to test anti-angiogenic compounds. It has the advantage of growing in a syngeneic mouse in which the host response, including the vasculature, is intact; is rapid, reproducible and metastatic [9].

Staurosporine (Fig. 1) is a non-selective protein kinase C inhibitor at low nanomolar concentrations [10] isolated from *Streptomyces* spp. by Omura et al. [11]. This alkaloid inhibits several ATP dependent kinases and phosphorylase kinases, and is able to interfere in the cell cycle via cyclin dependent kinases [12]. As most tyrosine kinases, along with many serine/threonine kinases, are either proto oncoproteins or involved in oncogenic signaling, the development of protein kinase inhibitors is a primary goal of cancer research. Staurosporine and many of its derivatives have significant biological effects, and these are being tested as anticancer drugs. For example, staurosporine is able to reduce VEGF-induced endothelial cell proliferation, due to PKC

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doi:10.1016/j.ejmech.2011.11.040

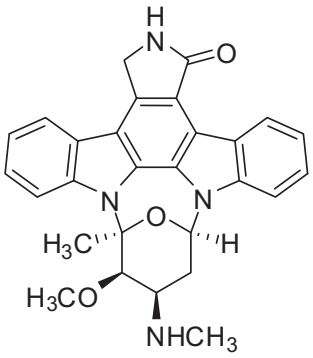


Fig. 1. Staurosporine.

implication in VEGF regulation. A disruption in VEGF signaling retards cancer progression, as tumor growth requires an expanding vasculature [13]. The main problem is the lack of selectivity of staurosporine, so interest is focused on the discovery or design of new analogs with more selective profiles [14]. Some compounds have been reported as anti-angiogenic but with more specific activity than staurosporine, as they have no or limited toxicity on endothelial cell culture [15]. In this sense, a desirable anti-angiogenic drug would inhibit vascular endothelial growth but not exhibit tumor cell antiproliferative activity, thus avoiding undesirable side effects.

The aim of this work was to explore the anti-angiogenic activity of a new series of indolocarbazole staurosporine analogs (Fig. 2, **2**). In previous works, we synthesized imidic *N*-substituted diphenylmaleimides (Fig. 2, **1**), and note that aminoalkyl side chains play an important role in the antiproliferative activity [16]. Therefore, a dialkylaminoalkyl group was attached to the imidic nitrogen.

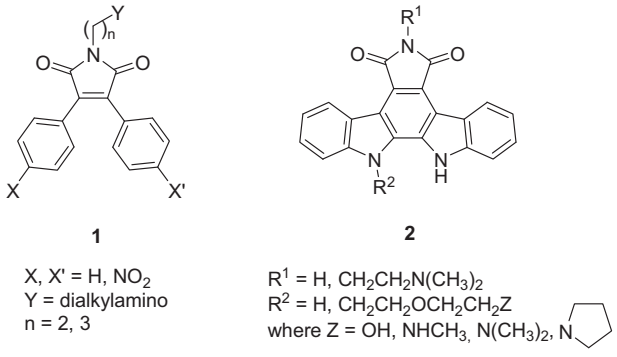


Fig. 2. (**1**) Imidic *N*-substituted diphenylmaleimides. (**2**) Indolocarbazole staurosporine analogs.

2. Results and discussion

2.1. Chemistry

Imide **3** was prepared from *N*-methyl-2,3-dibromomaleimide [17] and indolylmagnesium bromide following the procedure described by Faul et al. [18]. The compound **4** [19] was formed by the cyclization of **3**. Basic treatment of **4** led to anhydride **5** [20], direct precursor of **6** (Scheme 1).

To introduce the side chain in indol nucleus, imide **4**, was treated with 2-(2-iodoethoxy)ethyl acetate using cesium carbonate as base. Basic hydrolysis of imide **7** yield the anhydride **8**. Derivatives with a basic moiety in the imidic nitrogen were obtained by treatment of **8** with *N*,*N*-dimethylethylendiamine. Then, alcohol **9** was easily mesylated by reaction with methanesulfonyl chloride. Reaction of this intermediate with dimethylamine or methylamine led to the corresponding amines. Finally, the free amines were converted into their corresponding hydrochloride salts **10** and **11** by treatment with a saturated solution of hydrogen chloride in ethyl acetate (Scheme 2).

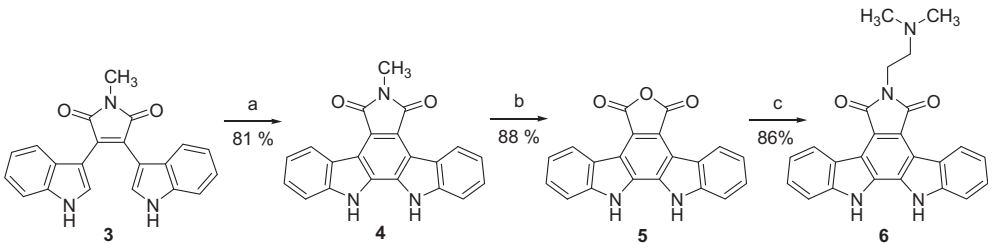
To obtain non-substituted indolocarbazole **12**, the anhydride **8** was converted into the imide derivative by reaction with HMDS in methanol, following the procedure described by Davis and Bit [21]. In addition to NH formation, silylation of the hydroxyl group was also observed, so that an additional treatment with TBAF was necessary to obtain **12**. Then, alcohol **12** was mesylated and then subjected to nucleophilic substitution reactions with dimethylamine or pyrrolidine to yield the desired amines. The amines were subsequently converted into their corresponding hydrochlorides to give **13** and **14** (Scheme 3).

2.2. In vitro and in vivo antiproliferative activity

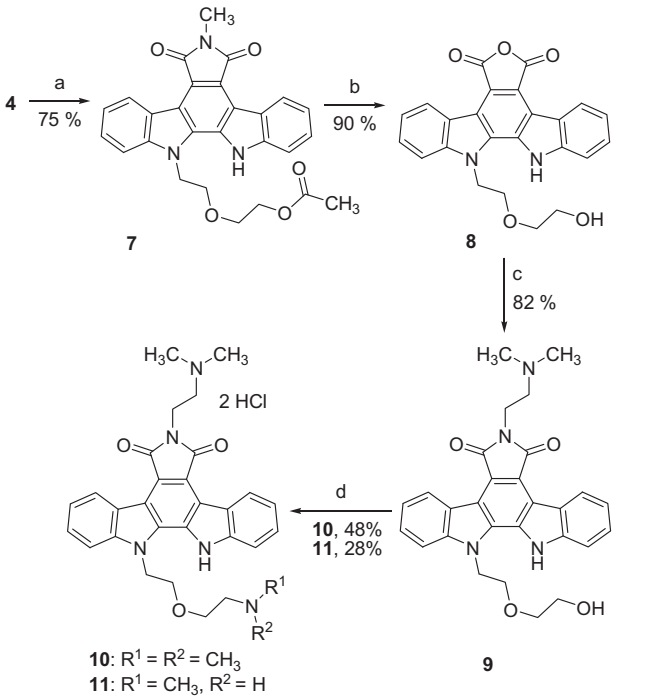
In order to study the potential anti-angiogenic activity of staurosporine analogs synthesized in this work, a preliminary screening was performed using antiproliferative activity measured by MTT assay. Human umbilical vein endothelial cell (HUVEC), human colon adenocarcinoma cell lines, LoVo and DLD-1, and Burkitt lymphoma cell line ST-486 were used in this study. Results are summarized in Table 1, and show compound selectivity in their antiproliferative activity. Suramin was used as the standard compound.

In those compounds with a dimethylaminoalkyl group as R^1, the presence of R^2 implies an improvement in activity and selectivity, noticeable with amine and alcoholic derivatives. These analogs showed similar submicromolar IC_{50}. Compound **10** was the most selective, being 200 times more active on HUVECs than on human colon cancer cell lines (Table 1). Indolocarbazoles with imidic NH were less selective, and the amine showed better antiproliferative activity on HUVECs than the alcohol.

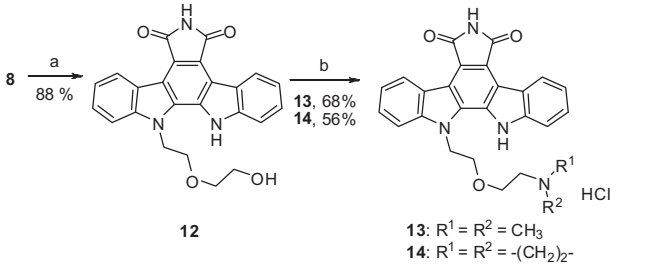
Comparing compounds **9**–**12** and **10**–**13**, we were able to confirm that the introduction of a dimethylaminoalkyl chain



Scheme 1. Reagents: (a) $CuCl_2$ (2 equiv), K_2CO_3 anh., 2-butanone, 80 °C; (b) (i) KOH 5 N, EtOH, reflux; (ii) 2 N HCl, 0 °C; (c) *N*,*N*-dimethylethylendiamine (2 equiv), DMF, 80 °C.



Scheme 2. Reagents: (a) Cs_2CO_3 (1 equiv), $CH_3CO_2CH_2CH_2OCH_2CH_2I$ (1 equiv), DMF, 60 °C; (b) (i) KOH 5 N, EtOH, reflux; (ii) 2 N HCl, 0 °C; (c) *N,N*-dimethylethylendiamine (2 equiv), DMF, 80 °C; (d) (i) MsCl (1.3 equiv), py, rt; (ii) 5% $NaHCO_3$, rt; (iii) R^1R^2NH (24 equiv), THF, rt; (iv) EtOAc sat. HCl g.



Scheme 3. Reagents: (a) (i) HMDS/MeOH (10:5), DMF, rt; (ii) TBAF, THF, rt; (b) (i) MsCl (2.6 equiv), py, rt; (ii) 5% $NaHCO_3$, rt; (iii) R^1R^2NH (24 equiv), THF, rt; (iv) EtOAc sat. HCl g.

considerably enhances activity and selectivity. It seems that imidic NH is not essential for antiproliferative activity and that, in this case, the substitution with a dimethylaminoethyl chain improves it.

All compounds were less active and, except for **6**, more selective than staurosporine as anti-angiogenic agents.

Table 1
Indolocarbazole antiproliferative activity.

Compound	HUVECs IC_{50} (μM)	LoVo IC_{50} (μM)	DLD-1 IC_{50} (μM)	ST-486 IC_{50} (μM)
6	7	0.50	3	1
9	0.1	0.3	20	3
10	0.1	20	20	4
11	0.3	6	7	3
12	10	4	2	0.1
13	2	–	–	2
Suramin	500	–	–	–
Staurosporine	0.004	0.001	0.009	0.007

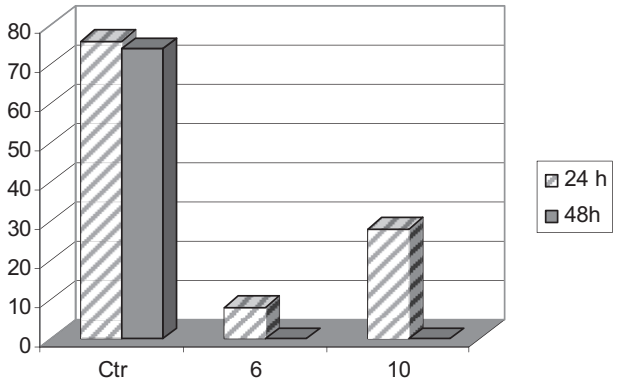


Fig. 3. Quantification of capillary-like structures during *in vitro* angiogenesis assay at 24 and 48 h of incubation.

With these results, **10** was selected to be tested in other *in vitro* anti-angiogenic assays as the most interesting compound due to its submicromolar antiproliferative activity and its excellent selectivity, besides it being water soluble. In order to determine the importance of R^2, compound **6** was also selected for further studies.

The effect of compounds **6** and **10** on HUVEC capillary-like structure formation in a 3D collagen gel, was used as a model to study *in vitro* angiogenesis. Results appear in Fig. 3.

At low concentrations, both compounds had the ability to inhibit capillary tube formation, showing complete inhibition at 48 h, but also a significant reduction with respect to the control at 24 h. *In vitro* assays developed in this study, point to **6** and **10** as promising anti-angiogenic compounds; thus they were selected for *in vivo* assays.

The effect of compounds **6** and **10** on tumor growth and metastasis was evaluated through the Lewis lung mice carcinoma model. Four animal groups were made for treatments. Weights of each animal group appear in Table 2. Results are shown in Table 3.

Even treatments with **6** and **10** showed no reduction in primary tumor size, however a slight reduction in metastasis number, process that depends on angiogenesis was observed.

Further studies with higher concentrations and other *in vivo* angiogenesis assays, as well as the preparation of new related derivatives are in progress.

Table 2
Animal weight (g).

	18th day		
	Mean	Median	SEM
G1 6	27.29	27.6	0.1
G2 10	28.5	28.2	0.0
G3 Suramin	25.5	25.8	0.1
G4 PBS	27.7	27.4	0.2

Table 3
Lewis lung carcinoma model results. Primary tumor weight (g) and lung metastasis number.

	Primary tumors weight				Lung metastasis number 1st observer			
	G1 6	**G2 10**	**G3 Sur**	**G4 PBS**	**G1 6**	**G2 10**	**G3 Sur**	**G4 PBS**
Mean	7.3	7.6	5.9	7.1	9.4	9.0	5.0	11.8
SD	1.5	1.4	0.9	1.1	6.3	8.7	3.6	7.1
SEM	0.4	0.3	0.2	0.3	1.5	2.0	0.9	1.7
Median	7.3	7.9	6.0	7.4	8.0	5.0	5.0	11.0

3. Conclusions

The synthesis and biological evaluation of a series of indolocarbazoles were carried out. In light of the biological activity assay results, we were able to conclude that the presence of dimethylaminoethyl chain in the imidic nitrogen, and the substitution of the indolic nitrogen enhance antiproliferative activity and selectivity.

4. Experimental protocols

4.1. Chemistry

Melting points (uncorrected) were determined on a Stuart Scientific SMP3 apparatus. Infrared (IR) spectra were recorded with a Perkin–Elmer 1330 infrared spectrophotometer. ^{1}H- and ^{13}C NMR were recorded on a Bruker 300-AC instrument. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (J) are in hertz. Elemental analyses (C, H, N) were performed on a Perkin–Elmer 2400 CHN apparatus at the Microanalyses Service of the University Complutense of Madrid; unless otherwise stated all reported values are within ±0.4% of the theoretical compositions. Thin-layer chromatography (TLC) was run on Merck silica gel 60 F-254 plates. Silica gel 60 of 230–400 mesh ASTM was used for column chromatography. Unless stated otherwise, starting materials used were high-grade commercial products. Compounds **4** and **5** have already been described and matched with the bibliography data [19,20].

4.1.1. Synthesis of 2-methyl-8,9-dihydro-1H-indolo [2,3-a]pyrrolo [3,4-c]carbazole-1,3(2H)-dione, *4*

To a stirred suspension of the 3,4-di(1*H*-indol-3-yl)-1-methyl-1*H*-pyrrole-2,5-dione, **3** (2.0 g, 5.87 mmol) and anhydrous K_2CO_3 (4.00 g) in 2-butanone (200 mL) was added 97% $CuCl_2$ (1.58 g, 11.73 mmol). The resulting mixture was heated at 80 °C for 2 h. Then, the crude was cooled to room temperature and filtered trough Celite. The organic layer was washed with 0.1 N HCl (3×100 mL), 5% $NaHCO_3$ (100 mL), water (100 mL) and brine (100 mL), dried with magnesium sulfate, filtered and solvents were removed under vacuo. The resulting solid was recrystallized from 2-butanone/EtOAc to afford **2** (1.61 g, 81%) as a yellow solid (mp > 330 °C). Compound **4** has already been described and the spectroscopic and physical data matched with the literature.

4.1.2. General procedure for the preparation of the anhydrides *5* and *8*

To a suspension of the corresponding imide (1.00 mmol) in EtOH (10 mL) was added 5 N KOH (45 mL). The reaction was refluxed for 2 h, cooled to 0 °C and concentrated HCl was added onto pH = 1. Then, the precipitate was collected by filtration and dissolved in H_2O. After overnight stirring at room temperature, the resulting suspension was filtered off to yield the corresponding anhydride.

*4.1.2.1. Synthesis of furo [3,4-c]indolo [2,3-a]carbazole-1,3(8H,9H)-dione, **5**.* Following the general procedure for the synthesis anhydrides, from 1.50 g (4.42 mmol) of **4**, 1.27 g (88%) of **5** were obtained as a yellow solid (mp > 250 °C). Compound **5** has already been described and the spectroscopic and physical data matched with the literature.

*4.1.2.2. Synthesis of 8-(2-(2-hydroxyethoxy)ethyl)furo [3,4-c]indolo [2,3-a]carbazole-1,3(8H,9H)-dione, **8**.* Following the general procedure for the synthesis anhydrides, from (0.77 g, 1.64 mmol) of **7**, 0.61 g (90%) of **8** were obtained as a yellow solid (mp > 250 °C). ^{1}H NMR (DMSO-d_6): δ 11.98 (s, 1H, NH), 8.86 (d, 1H, Ar, J = 8.0 Hz), 8.83 (d, 1H, Ar, J = 8.0 Hz), 7.85 (d, 1H, Ar, J = 8.6 Hz), 7.82 (d, 1H, Ar, J = 8.6 Hz), 7.66–7.57 (m, 2H, Ar), 7.43–7.35 (m, 2H, Ar), 5.04 (t, 2H, CH_2, J = 4.9 Hz), 4.49 (t, 1H, OH, J = 5.2 Hz), 3.94 (t, 2H, CH_2, J = 4.9 Hz), 3.30 (t, 2H, CH_2, J = 5.2 Hz), 3.24–3.19 (m, 2H, CH_2). ^{13}C NMR (DMSO-d_6): δ 164.7, 164.6, 141.2, 140.8, 130.9, 129.3, 127.4, 127.3, 123.5, 123.3, 121.0, 120.8, 120.6, 120.3, 117.5, 117.5, 116.9, 115.9, 112.4, 110.7, 72.4, 69.6, 59.8, 44.9. IR (KBr): 3600–3200 (OH, NH), 1810 (C=O), 1740 (C=O) cm^{-1}.

4.1.3. General procedure for the preparation of the imides *6* and *9*

A mixture of the corresponding anhydride (0.10 mmol) and *N*,*N*-dimethylethylendiamine (0.20 mmol) in dry DMF (2 mL) was heated at 80 °C for 20 h. Then, the crude was cooled to room temperature and the solvent was removed under reduced pressure. The resulting residue was purified by flash chromatography or recrystallization to give the corresponding imide.

*4.1.3.1. Synthesis of 2-(2-(dimethylamino)ethyl)-8,9-dihydro-1H-indolo [2,3-a]pyrrolo [3,4-c]carbazole-1,3(2H)-dione, **6**.* Following the general procedure for the synthesis of imides, from 0.20 g (0.61 mmol) of **5** and 0.13 mL (1.23 mmol) of *N*,*N*-dimethylethylendiamine, and after the residue was collected by filtration, disgregated in H_2O and filtered, 0.21 g (86%) of **6** were obtained as a yellow solid (mp > 250 °C). ^{1}H NMR (DMSO-d_6): δ 11.79 (bs, 2H, 2× NH), 9.00 (d, 2H, Ar, J = 7.3 Hz), 7.83 (d, 2H, Ar, J = 7.9 Hz), 7.57 (t, 2H, Ar, J = 7.6 Hz), 7.36 (t, 2H, Ar, J = 7.6 Hz), 3.82 (t, 2H, CH_2, J = 6.4 Hz), 2.61 (t, 2H, CH_2, J = 6.1 Hz), 2.23 (s, 6H, 2× CH_3). ^{13}C NMR (DMSO-d_6): δ 169.8, 140.4, 129.0, 127.0, 124.3, 121.5, 120.3, 118.7, 115.7, 112.2, 56.9, 45.0, 35.1. IR (KBr): 3460 (broad, NH), 1750 (C=O), 1700 (C=O) cm^{-1}. Anal. calcd for $C_{24}H_{20}N_4O_2$: C, 72.71; H, 5.08; N, 14.13. Found: C, 72.69; H, 5.16, N, 14.06.

*4.1.3.2. Synthesis of 2-(2-(dimethylamino)ethyl)-8-(2-(2-hydroxtyethoxy)ethyl)-8,9-dihydro-1H-indolo [2,3-a]pyrrolo [3,4-c]carbazole-1,3(2H)-dione, **9**.* Following the general procedure for the synthesis of imides, from 0.10 g (0.97 mmol) of **8** and 0.20 mL (1.94 mmol) of *N*,*N*-dimethylethylendiamine, and after purification by flash chromatography ($CHCl_3$:MeOH, 95:5), 0.39 g (82%) of **9** were obtained as a yellow solid (mp > 170 °C, dec. $CHCl_3$/MeOH). ^{1}H NMR (DMSO-d_6): δ 11.84 (bs, 1H, NH), 9.12 (d, 1H, Ar, J = 7.9 Hz), 9.09 (d, 1H, Ar, J = 8.5 Hz), 7.82 (d, 2H, Ar, J = 8.0 Hz), 7.64–7.55 (m, 2H, Ar), 7.42–7.34 (m, 2H, Ar), 5.08 (bs, 2H, CH_2), 4.53 (bs, 1H, OH), 3.95 (bs, 2H, CH_2), 3.79 (t, 2H, CH_2, J = 6.4 Hz), 3.31 (m, 2H, CH_2), 3.24 (bs, 2H, CH_2), 2.59 (t, 2H, CH_2, J = 6.1 Hz), 2.22 (s, 6H, 2× CH_3). ^{13}C NMR (DMSO-d_6): δ 169.4, 141.4, 140.9, 130.1, 128.5, 126.8, 126.7, 124.3, 124.1, 121.0, 120.8, 120.3, 120.1, 118.5, 118.5, 116.9, 115.9, 112.0, 110.2, 72.3, 69.6, 59.8, 56.9, 45.1, 44.9, 35.3. IR (KBr): 3380 (broad, OH, NH), 1740 (C=O), 1690 (C=O) cm^{-1}. Anal. calcd for $C_{28}H_{28}N_4O_4$: C, 69.41; H, 5.82; N, 11.56. Found: C, 69.52; H, 5.91, N, 11.50.

4.1.4. Synthesis of 2-(2-(2-methyl-1,3-dioxo-2,3-dihydro-1H-indolo [2,3-a]pyrrolo [3,4-c]carbazol-8(9H)-yl)ethoxy)ethyl acetate, *7*

To a solution of the imide **4** (0.83 g, 2.45 mmol) and Cs_2CO_3 (0.80 g, 2.45 mmol) in dry DMF (30 mL) was added a solution of 2-(2-iodoethoxy)ethyl acetate (0.63 g, 2.45 mmol) in dry DMF (20 mL) and the reaction was stirred at 60 °C for 16 h under Ar. The solution was cooled to room temperature and 100 mL of EtOAc was then added. The organic phase was washed with water (2 × 100 mL). The aqueous phase was extracted with EtOAc (2 × 100 mL) and the organic layer was dried over magnesium sulfate, evaporated to dryness in vacuo to obtain a crude which was purified by flash chromatography (toluene/dioxane, 95:5); 0.20 g (75%) of compound **7** were obtained as a yellow solid (mp = 196–197 °C, dioxane). ^{1}H NMR ($CDCl_3$): δ 10.12 (s, 1H, NH), 9.16 (d, 1H, Ar, J = 7.7 Hz), 9.12 (d, 1H, Ar, J = 8.3 Hz), 7.56–7.47 (m, 3H, Ar),

7.43–7.26 (m, 2H, Ar), 7.23 (d, 1H, Ar, $J = 7.7$ Hz), 4.56 (t, 2H, CH_2, $J = 4.4$ Hz), 4.08–4.04 (m, 4H, 2× CH_2), 3.65–3.62 (m, 2H, CH_2), 3.00 (s, 3H, CH_3N), 1.47 (s, 3H, CH_3CO). ^{13}C NMR ($CDCl_3$): δ 170.7, 169.7, 169.5, 141.1, 141.0, 130.4, 129.2, 126.9, 126.7, 125.6, 125.3, 122.2, 121.8, 120.9, 120.7, 119.2, 119.1, 118.0, 116.4, 111.1, 108.0, 70.5, 69.5, 62.7, 45.5, 23.1, 20.1. IR (KBr): 3390 (NH), 1750 (C═O), 1710 (C═O), 1690 (C═O) cm^{-1}. Anal. calcd for $C_{27}H_{23}N_3O_5$: C, 69.07; H, 4.94; N, 8.95. Found: C, 69.11; H, 5.01, N, 9.11.

4.1.5. Synthesis of 8-(2-(2-hydroxyethoxy)ethyl)-8,9-dihydro-1H-indolo [2,3-a]pyrrolo [3,4-c]carbazole-1,3(2H)-dione, **12**

To a suspension of the anhydride **8** (0.47 g, 1.13 mmol) in dry DMF (7 mL) was added a mixture of hexamethyldisilazane (2.35 mL, 11.3 mmol) and methanol (0.23 mL, 5.65 mmol) and the reaction was stirred at room temperature for 22 h. After completing the reaction, 50 mL of H_2O were added. The mixture was extracted with EtOAc (3 × 50 mL) and the organic layers were washed with water (50 mL), dried over magnesium sulfate and evaporated under reduced pressure giving a crude mixture containing **12** and its silylated derivative. Separation by flash chromatography (hexane/EtOAc, 3:1 to EtOAc) afforded pure **12** (0.23 g) as yellow solid (mp = 189–191 °C) and its silylated derivative (0.21 g). The silylated derivative was dissolved in THF (6 mL) and was treated with 0.75 mL (0.82 mmol) of tetrabutylammonium fluoride (1.1 M in THF) and the reaction was stirred at room temperature for 16 h in an argon atmosphere. After completing the reaction, 15 mL of H_2O were added. The mixture was extracted with EtOAc (25 mL) and the organic layers were washed with water (15 mL), dried over magnesium sulfate and evaporated under reduced pressure to yield 0.18 g of pure **12** (88%). ^{1}H NMR (DMSO-d_6): δ 11.75 (bs, 1H, NH), 11.08 (bs, 1H, NH) 9.12 (d, 1H, Ar, $J = 8.6$ Hz), 9.09 (d, 1H, Ar, $J = 8.5$ Hz), 7.84 (d, 1H, Ar, $J = 8.6$ Hz), 7.80 (d, 1H, Ar, $J = 8.0$ Hz), 7.61 (t, 1H, Ar, $J = 7.6$ Hz), 7.57 (t, 1H, Ar, $J = 7.6$ Hz), 7.41–7.34 (m, 2H, Ar), 5.09 (t, 2H, CH_2, $J = 4.9$ Hz), 4.52 (bs, 1H, OH), 3.97 (t, 2H, CH_2, $J = 4.6$ Hz), 3.32 (m, 2H, CH_2), 3.26 (t, 2H, CH_2, $J = 4.9$ Hz). ^{13}C NMR (DMSO-d_6): δ 171.1, 171.1, 141.4, 140.9, 130.2, 128.6, 126.9, 126.8, 124.5, 124.3, 121.1, 120.9, 120.4, 120.2, 119.8, 116.9, 115.9, 112.1, 110.3, 72.4, 69.7, 59.9, 44.91. IR (KBr): 3500, 3400, 3300, 3200 (broad, OH, NH), 1745 (C═O), 1680 (C═O) cm^{-1}. Anal. calcd for $C_{24}H_{19}N_3O_4$: C, 69.72; H, 4.63; N, 10.16. Found: C, 69.67; H, 4.71, N, 10.01.

Data of 8-(2-(2-(*trimethylsilyloxy*)*ethoxy*)*ethyl*)-8,9-*dihydro*-1*H*-*indolo* [2,3-*a*]*pyrrolo* [3,4-*c*]*carbazole*-1,3(2*H*)-*dione*. ^{1}H NMR (DMSO-d_6): δ 11.79 (s, 1H, NH) 11.01 (s, 1H, NH), 9.11 (d, 1H, Ar, $J = 8.6$ Hz), 9.08 (d, 1H, Ar, $J = 8.6$ Hz), 7.81 (d, 1H, Ar, $J = 9.1$ Hz), 7.78 (d, 1H, Ar, $J = 9.1$ Hz), 7.61–7.53 (m, 2H, Ar), 7.39–7.32 (m, 2H, Ar), 5.10 (t, 2H, CH_2, $J = 4.6$ Hz), 3.93 (t, 2H, CH_2, $J = 4.9$ Hz), 3.27–3.22 (m, 4H, 2× CH_2), −0.28 (s, 9H, 3× CH_3Si). ^{13}C NMR (DMSO-d_6): δ 171.2, 171.2, 141.4, 141.0, 130.3, 128.7, 127.0, 126.9, 124.5, 124.3, 121.2, 121.0, 120.5, 120.3, 119.9, 116.9, 115.9, 112.2, 110.4, 72.5, 69.7, 59.9, 45.0, 8.8, 8.6. IR (KBr): 3280 (NH), 3200 (broad, NH), 1740 (C═O), 1690 (C═O), 1010 (Si–O), 800 (Si–O) cm^{-1}

4.1.6. General procedure for the preparation of the hydrochlorides **10**, **11**, **13** and **14**

To a solution of the corresponding alcohol (2.0 mmol) in dry pyridine (3.5 mL) methanesulfonyl chloride was added dropwise while the temperature was maintained below 0 °C. The reaction was stirred at room temperature for 90 min and 20 mL of 5% $NaHCO_3$ were added. The layers were separated, the aqueous phase was extracted with $CHCl_3$ (100 mL) and the organic phase was washed with brine (25 mL), dried over magnesium sulfate and evaporated under reduced pressure giving the corresponding mesylate derivative which was used without further purification. Then, to a suspension of the mesylate derivative in THF (20 mL) was added the corresponding amine (24 mmol) and the reaction was stirred at room temperature. The reaction mixture was diluted with 50 mL of dichloromethane and washed with 40 mL of water, dried over magnesium sulfate and evaporated under reduced pressure. The residue was purified by flash chromatography (chloroform/methanol, 95:5) to afford the pure amino derivative. This compound was dissolved in EtOAc (100 mL) and 14 mL of saturated EtOAc/HCl (g) solution were added. After stirring for 1 h at room temperature, the resulting suspension was filtered off to yield the corresponding hydrochloride.

4.1.6.1. Synthesis of 8-(2-(2-(dimethylamino)ethoxy)ethyl)-2-(2-(dimethylamino)ethyl)-8,9-dihydro-1H-indolo [2,3-a]pyrrolo [3,4-c] carbazole-1,3(2H)-dione dihydrochloride, **10**.

Following the general procedure for the synthesis of hydrochlorides, from 1.10 g (2.27 mmol) of **9**, 0.22 mL (2.95 mmol) of methanesulfonyl chloride and 6.13 mL (54.48 mmol) of dimethylamine (40% aqueous solution), after stirring for 90 min, 0.61 g (48%) of **10** were obtained as a yellow solid (mp > 250 °C). ^{1}H NMR (DMSO-d_6): δ 12.39 (s, 1H, NH), 10.45 (bs, 1H, NH^+), 10.11 (bs, 1H, NH^+), 9.13 (d, 1H, Ar, $J = 7.9$ Hz), 9.09 (d, 1H, Ar, $J = 7.9$ Hz), 8.03 (d, 1H, Ar, $J = 8.6$ Hz), 7.90 (d, 1H, Ar, $J = 8.6$ Hz), 7.55–7.65 (m, 2H, Ar), 7.41 (t, 1H, Ar, $J = 7.6$ Hz), 7.37 (t, 1H, Ar, $J = 7.3$ Hz), 5.28 (bs, 2H, CH_2), 4.10 (t, 2H, CH_2, $J = 5.5$ Hz), 3.96 (bs, 2H, 2× CH_2), 3.50–3.56 (m, 4H, 2× CH_2), 3.04 (bs, 2H, CH_2), 2.92 (d, 6H, 2× CH_3, $J = 3.7$ Hz), 2.49 (bs, 6H, 2× CH_3). ^{13}C NMR (DMSO-d_6): δ 169.7, 141.1, 141.1, 130.2, 128.2, 127.0, 126.9, 124.4, 124.1, 121.1, 120.6, 120.5, 120.2, 118.9, 118.8, 116.9, 115.9, 112.4, 110.5, 68.9, 64.4, 54.9, 54.8, 44.4, 42.3, 42.1, 32.8. IR (KBr): 2700–2340 (NH^+), 1745 (C═O), 1690 (C═O) cm^{-1}. Anal. calcd for $C_{30}H_{35}Cl_2N_5O_3$: C, 61.64; H, 6.04; N, 11.98. Found: C, 61.72; H, 5.99, N, 11.82.

4.1.6.2. Synthesis of 2-(2-(dimethylamino)ethyl)-8-(2-(2-(methylamino)ethoxy)ethyl)-8,9-dihydro-1H-indolo [2,3-a]pyrrolo [3,4-c] carbazole-1,3(2H)-dione dihydrochloride, **11**.

Following the general procedure for the synthesis of hydrochlorides, from 0.20 g (0.42 mmol) of **9**, 0.04 mL (0.53 mmol) of methanesulfonyl chloride and 0.87 mL (10.08 mmol) of methylamine (40% aqueous solution), after stirring for 18 h, 0.07 g (28%) of **11** were obtained as a yellow solid (mp > 180 °C, dec.). ^{1}H NMR (DMSO-d_6): δ 12.40 (s, 1H, NH), 10.08 (bs, 1H, NH^+), 9.14 (d, 1H, Ar, $J = 7.9$ Hz), 9.09 (d, 1H, Ar, $J = 7.9$ Hz), 9.05 (bs, 2H, NH_2^+), 8.05 (d, 1H, Ar, $J = 7.9$ Hz), 7.90 (d, 1H, Ar, $J = 8.6$ Hz), 7.66–7.56 (m, 2H, Ar), 7.43–7.34 (m, 2H, Ar), 5.27 (bs, 2H, CH_2), 4.10 (t, 2H, CH_2, $J = 5.2$ Hz), 3.96 (bs, 2H, CH_2), 3.50 (bs, 4H, 2× CH_2), 2.92 (s, 6H, 2× CH_3), 2.86 (bs, 2H, CH_2), 2.36 (bs, 3H, CH_3). ^{13}C NMR (DMSO-d_6): δ 169.7, 141.1, 130.2, 128.2, 127.0, 124.4, 124.0, 121.0, 120.6, 120.5, 120.2, 118.9, 118.8, 116.9, 115.9, 112.5, 110.5, 68.9, 65.4, 54.9, 46.8, 44.5, 42.3, 32.9, 32.2. IR (KBr): 2820–2350 (NH_2^+, NH^+), 1745 (C═O), 1690 (C═O) cm^{-1}. Anal. calcd for $C_{29}H_{33}Cl_2N_5O_3$: C, 61.05; H, 5.83; N, 12.28. Found: C, 61.12; H, 5.91, N, 12.11.

4.1.6.3. Synthesis of 8-(2-(2-(dimethylamino)ethoxy)ethyl)-8,9-dihydro-1H-indolo [2,3-a]pyrrolo [3,4-c]carbazole-1,3(2H)-dione hydrochloride, **13**.

Following the general procedure for the synthesis of dimethylamine hydrochlorides, from 0.20 g (0.48 mmol) of **12**, 0.1 mL (1.26 mmol) of methanesulfonyl chloride and 0.22 mL (11.52 mmol) of dimethylamine (40% aqueous solution), after stirring for 2 h, 0.16 g (68%) of **13** were obtained as a yellow solid (mp > 250 °C). ^{1}H NMR (DMSO-d_6): δ 12.21 (s, 1H, NH), 11.04 (s, 1H, NH), 10.11 (bs, 1H, NH^+), 9.14 (d, 1H, Ar, $J = 7.3$ Hz), 9.09 (d, 1H, Ar, $J = 7.9$ Hz), 7.97 (d, 1H, Ar, $J = 7.9$ Hz), 7.88 (d, 1H, Ar, $J = 8.5$ Hz), 7.64–7.54 (m, 2H, Ar), 7.39 (t, 1H, Ar, $J = 7.3$ Hz), 7.36 (t, 1H, Ar, $J = 7.6$ Hz), 5.26 (bs, 2H, CH_2), 3.96 (bs, 2H, CH_2), 3.57 (bs, 2H, CH_2), 3.02 (bs, 2H, CH_2), 2.46 (s, 6H, 2× CH_3). ^{13}C NMR (DMSO-d_6): δ 171.2, 171.2, 141.0, 140.9, 130.2, 128.3, 126.8, 126.7, 124.6, 124.3,

121.2, 120.8, 120.4, 120.2, 119.9, 119.8, 116.8, 115.8, 112.1, 110.4, 69.1, 64.5, 55.1, 44.4, 42.2. IR (KBr): 3260, 3200 (broad NH), 2700–2500 (NH^+), 1740 (C=O), 1710 (C=O) cm^{-1}. Anal. calcd for $C_{26}H_{25}ClN_4O_3$: C, 65.47; H, 5.28; N, 11.75. Found: C, 65.56; H, 5.22, N, 11.81.

4.1.6.4. Synthesis of 8-(2-(2-(pyrrolidin-1-yl)ethoxy)ethyl)-8,9-dihydro-1H-indolo [2,3-a]pyrrolo [3,4-c]carbazole-1,3(2H)-dione hydrochloride, ***14****.* Following the general procedure for the synthesis of hydrochlorides, from 0.17 g (0.41 mmol) of **12**, 0.08 mL (1.07 mmol) of methanesulfonyl chloride and 0.82 mL (9.84 mmol) of pyrrolidine, after stirring for 3 h, 0.12 g (56%) of **14** were obtained as a yellow solid (mp > 250 °C). ^{1}H NMR (DMSO-d_6): δ 12.20 (s, 1H, NH), 11.06 (s, 1H, NH), 10.10 (bs, 1H, NH), 9.14 (d, 1H, Ar, J = 7.9 Hz), 9.10 (d, 1H, Ar, J = 8.0 Hz), 7.93 (d, 1H, Ar, J = 7.9 Hz), 7.88 (d, 1H, Ar, J = 8.0 Hz), 7.55–7.65 (m, 2H, Ar), 7.40 (t, 1H, Ar, J = 7.6 Hz), 7.36 (t, 1H, Ar, J = 7.6 Hz), 5.27 (bs, 2H, CH_2), 3.94 (bs, 2H, CH_2), 3.50 (bs, 2H, CH_2), 3.32 (m, 2H, CH_2), 3.01 (bs, 3H, 2× CH_2), 2.51 (bs, 1H, CH_2), 1.61 (bs, 4H, 2× CH_2). ^{13}C NMR (DMSO-d_6): δ 171.2, 171.2, 141.0, 140.9, 130.3, 128.4, 126.9, 126.8, 124.6, 124.3, 121.3, 120.8, 120.4, 120.3, 119.9, 119.8, 116.8, 115.8, 112.0, 110.4, 69.1, 65.8, 53.1, 52.6, 44.4, 22.3. IR (KBr): 3260, 3170 (broad NH), 2710–2420 (NH^+), 1735 (C=O), 1700 (C=O) cm^{-1}. Anal. calcd for $C_{28}H_{27}ClN_4O_3$: C, 66.86; H, 5.41; N, 11.14. Found: C, 66.76; H, 5.35, N, 11.23.

4.2. Biological assays

4.2.1. MTT assay

Cells were seeded in 96-well culture plates at 3000 cells (HUVECs) or 10,000 (tumors) per well in normal medium. 24 h later, serial dilutions of the compounds were added and plates were incubated for a further 48–72 h. During the last 4 h, plates were incubated with 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2-5-diphenyl-2*H*-tetrazolium bromide (MTT, Merck KgaA) at 5 mg/mL. Finally, 100 μL of extraction buffer (20% sodium dodecyl sulfate and 50% sodium *N*,*N*-dimethylformamide, pH 4.7) were added and plates were incubated for 18 h at 37 °C. Readings were taken in a microtiter plate spectrophotometer at 570 and 630 nm to eliminate the background.

4.2.2. In vitro angiogenesis assay

HUVECs were cultured in Endothelial Basal Medium (EBM) supplemented with 2% Fetal Bovine Serum (FBS), 50 mg/mL gentamicin sulfate, 1 mg/mL hydrocortisone, 10 mg/mL recombinant human Epidermal Growth Factor (hEGF), 3 mg/mL Bovine Brain Extract (BBE), and 10% Fetal Calf Serum (FCS). Confluent cultures were harvested with 0.05% trypsin/0.53 mM EDTA and plated in a 1:2–1:3 relation. HUVEC cells from passages 5–11 were used for this study. A gel of rat tail type I collagen was used as cell support. Cells were seeded at 1×10^5 cells/mL density in a 3D collagen matrix (1.2 mg/mL) and EBM medium supplemented with 100 U/mL heparin and 2.5 ng/mL basic Fibroblast Growth Factor (BFGF). Compounds were added at concentrations of 20 μM. The number of capillary-like structures was quantified per area.

4.2.3. Lewis lung mice carcinoma in vivo model

Cells: LL2 cell line was cultured in RPMI-1640 supplemented with 10% FCS and 2 mM Glutamax-I in 150 cm^2 flasks. Cells were kept in exponential growth. Mice: males C57BL/6 mice (HARLAM, Spain) with weight between 19 and 21 g (6 weeks old). Animals were randomly selected before treatment. Model: Cells were collected, washed twice with Earle's-BSS, counted and adjusted to 20×10^6 cells/mL in EBSS. Cell viability remained higher than 95%. Cells were intramuscularly injected into the right hind leg of mice (2×10^6 cells/mouse). After administration, cell viability remained higher than 80%. Animals were sacrificed 17–21 days after inoculation. Treatment: **6** and **10** compounds were diluted to 5 μg/mL in PBS. Suramin was diluted to 4 mg/mL in PBS. Products were administered daily 24 h after LL2 cell injection. Compounds and vehicle were injected intraperitoneally at a dosage of 100 μL to each animal.

Acknowledgments

We thank Dirección General de Estudios Superiores (Gant No. PB98-055, CYTED), Laboratorios Knoll (BASF Pharma), Universidad CEU-San Pablo for financial support and Brian Crilly for linguistic assistance. L. Añorbe acknowledges Comunidad de Madrid for a predoctoral fellowship, Ministerio de Educación y Ciencia (BFU 2004-02838/BFI).

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