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Targeting the colchicine site in tubulin through cyclohexanedione derivatives

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TITLE RUNNING HEAD: Colchicine-site binders based on cyclohexanediones

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ABSTRACT.

Cyclohexanedione derivatives represent a new family of colchicine-site binders that were identified through a ligand-based virtual screening approach. Structural modifications have now been performed at both distal sites of our identified hit [2-(1-((2-methoxyphenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (**4**)] in order to improve tubulin binding affinity, anti-proliferative activity and/or aqueous solubility. The results obtained indicate that the 2-methoxyphenyl ring, the fragment located closer to the αβ-tubulin interface according to docking studies, is the one that allows structural variation in order to improve the Ka value against tubulin (as in compound **20a** with a Ka= $1.3x10^7$ M^{-1}, analogous to colchicine) or to improve aqueous solubility, as in compound **22c**, being more than 10-times more soluble than the previous hit **4**.

KEYWORDS: αβ-tubulin; colchicine; vascular-disrupting agents; cyclohexanediones.

Introduction

Microtubules (MT) are key components of the cytoskeleton and play a crucial role in different cellular processes, such as cell motility, morphogenesis and mitosis.[1] MT are highly dynamic and are composed of αβ-tubulin heterodimers. Suppression of MT dynamics blocks the cell division machinery at mitosis leading to cell death. Several drugs have been described to interact with the αβ-tubulin heterodimer to stabilize (taxanes and laulimalides) or destabilize (vinca alkaloids and colchicine) the polymerization process of tubulin into microtubules.[2] Therefore, MT dynamics constitute a validated target for tumor therapy. In addition, compounds that bind at the colchicine-site are being deeply explored in antivascular therapies, as an adjuvant in anticancer treatment.[3, 4] Colchicine itself (**1**) has been discarded as an anticancer agent due to its toxicity, but compounds that target the αβ–tubulin dimer at the colchicine-binding site, such as combretastatin A-4 (CA-4, **2a**) or its related derivatives CA-4P (**2b**) and AVE8062 (**2c**) (Figure 1) have been proposed as valuable anticancer agents since they combine antimitotic properties with vascular disrupting capacity, targeting tumor and endothelial cells, respectively [5, 6]

The colchicine site at the αβ-tubulin interface is able to adapt quite a variety of structurally unrelated ligands, and is commonly referred to as the "colchicine-binding domain".[7, 8] Thus novel ligands can be identified based on structural information of this domain. Using the coordinates of TN-16 (**3**, Figure 1) obtained from its X-Ray complex with αβ-tubulin,[9] and following a ligand-based virtual screening approach, we have recently described a family of cyclohexanediones, that represents a novel class of colchicine-site binders.[10] The most representative compound (**4**, Figure 1) was shown to inhibit tumor and endothelial cell proliferation in the sub-μM range. Its mechanism of action involves cell cycle arrest in the G2/M phase and an increase in apoptosis. Compound **4** was also shown to destroy an established endothelial tubular network and to inhibit the migration and invasion of human breast carcinoma cells.[10] Therefore, this new family of colchicine-site binders, exemplified by compound **4**, has shown significant therapeutic potential.

The structural requirements for anti-proliferative activity established in our previous paper[10] among this series of cyclohexanediones can be summarized as follows: (i) fragment **A** (Figure 2) should be an aromatic ring, preferentially unsubstituted; (ii) the alkyl chain at fragment **C** plays an important role in the anti-proliferative activity, in the order Me>Et>>>Pr; (iii) substitutions at the aromatic ring **D** at *para* or *meta* position lead to poorly active compounds, while substitutions at *ortho* render active compounds, the best results, among the substituents assayed, were obtained with a methoxy or a methyl.

Our objective in this paper has been to improve the low aqueous solubility (< 10 μM) of our initial series of compounds. With this purpose we have undertaken the synthesis of new structural analogues by incorporating novel substituents at fragments **A**, **C** or **D** of our lead compound **4** (Figure 2) as follows: 1) the phenyl ring at fragment **A** has been replaced by aromatic heterocycles; 2) a CH_2OH group has been introduced at fragment **C** replacing the methyl of the lead compound, and 3) a wide variety of substitutions has been introduced at the *ortho* position of fragment **D** by incorporating polar groups, by elongating the substituent and/or by incorporating solubilizing groups. Based on docking studies, also here reported, this fragment **D** faces the αβ-tubulin interface, being closer to the solvent-exposed area. In all cases, the central core of the molecule has been kept intact since this determines the overall conformation of these compounds making them suitable to bind similarly to TN-16 at the αβ-tubulin interface as initially designed. Therefore we here report on the design and synthesis of a second series of cyclohexanedione derivatives, their antiproliferative activity against tumor and endothelial cell lines and their effect on the cell cycle. For the most relevant compounds, aqueous solubility has been experimentally measured and binding to the colchicine-site has been investigated by competition experiments. Finally, the results obtained have been rationalized based on docking studies.

Results and Discussion

As mentioned in the introduction, the first series of modifications addressed in this paper involved replacement of the phenyl ring on fragment **A** in compound **4** by heteroaromatic rings such as pyridines, thiophene or furane. Thus, reaction of the pyridylbutenones (**5a-c**) with diethylmalonate in the presence of sodium ethoxide (Scheme 1) afforded the corresponding cyclohexane-1,3-diones **6a-c** that were heated with acetyl chloride in the presence of anhydrous K_2CO_3, 1,2,4-triazole and tetrabutylammonium bromide in anhydrous DMF, as previously setup[10], obtaining the corresponding 2-acetylcyclohexanediones **7a-c** in good to moderate yields. These compounds (**7a-c**) and the corresponding thiophenyl (**7d**) or furanyl (**7e**) derivatives, that were synthesized following described procedures,[11] reacted with *o*-anisidine in toluene at 110 °C overnight to afford the condensation products **8a-e** in good to excellent yields (44-99%). Next the methyl group at fragment **C** in compound **4** was replaced by an hydroxymethyl group. Thus, reaction of the commercially available 5-phenylcyclohexane-1,3-dione (**9**) (Scheme 2) with acetoxyacetylchloride in dichloromethane in the presence of triethylamine and DMAP[12] afforded the *C*-acylderivative (**10**) in 68% yield. Treatment of **10** with *o*-anisidine in toluene at 110 °C afforded **11a** that was deacetylated to provide the hydroxymethyl derivative **11b**.

A wider set of modifications were performed at fragment **D**. Introduction of an hydroxymethyl group at the *ortho* position of the phenyl ring (Scheme 3) was carried out by reaction of the 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] with 2-(((*tert*-butyldimethylsily)oxy)methyl)aniline (**13**)[12] followed by treatment of the condensation product with TBAF to afford compound **14** in 73% yield. Similarly, reaction of the 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**) with 2-aminopyridine (**15**) in toluene at 110 °C overnight provided the 2-pyridyl derivative **16** in 84% yield. Alternatively, reaction of **12** with *o*-phenylendiamine (**17a**) or *N*-methyl-1,2-benzenediamine (**17b**) in toluene afforded the condensation products **18a** and **18b** in 83 and 76% yield, respectively. A small series of alkoxy groups different to the OMe in compound **4** were also incorporated at fragment **D**. Thus, reaction of the acylcyclohexanedione **12** with the corresponding anilines **19a-d** (Scheme 3) in toluene gave access to the 2-ethoxy, 2-propoxy, 2-isopropxy and 2-cyclopropylmethoxy derivatives **20a-d**, respectively. Compounds **20a-d** showed interesting anti-proliferative activity (see Biological evaluation section), indicating that longer substitutions at position 2 in ring **B** could be envisaged. Therefore, a small series of glycol derivatives with a terminal polar group was envisioned meant to reach the solvent exposed area at the interface while increasing solubility. A similar approach has been successfully used in the kinases field.[13] Thus reaction of **12** with 2-(2-((*tert*-butyldimethylsilyl)oxy)ethoxy)aniline **21a**[14] (Scheme 4) or its propoxy analoge **21b**[14, 15] afforded the corresponding condensation products that were treated with TBAF to remove the silyl group. In this way compounds **22a** and **22b** were obtained. Similarly, reaction of **12** with 2-(2-(2-((*tert*-butyldimethylsilyl)oxy)ethoxy)ethoxy)aniline (**21c**)[14, 15] or *tert*-butyl(2-(2-(2-aminophenoxy)ethoxy)ethyl)carbamate (**21d**),[16] and subsequent treatment with TBAF or TFA afforded compounds **22c** and **22d** in 57 and 84% yield, respectively.

Since the incorporation of morpholinyl and piperidyl substituents has also been proposed as a good alternative to improve solubility,[17, 18] such groups were incorporated at the *ortho* position at ring **D** as shown in Scheme 5. Thus, reaction of **12** with 2-[2-(morpholin-4-yl)ethoxy]aniline (**23**),[19] afforded the morpholinoethoxy derivative **24** in 32% yield. On the other hand, reaction of **12** with 2-((1-(3-((*tert*-butyldimethylsilyl)oxy)propyl)piperidin-4-yl)oxy)aniline (**25**) followed by silyl deprotection provided derivative **26.**

Biological evaluation

Anti-proliferative activity

The synthesized compounds were evaluated for their anti-proliferative activity in five different cell lines: two endothelial cell lines [human microvascular endothelial cells (HMEC-1) and bovine aortic endothelial cells (BAEC)] and three cancer cell lines [mouse lymphocytic leukemia (L1210), human lymphoblastic leukemia (CEM) and human cervical carcinoma (HeLa) cells]. Data are expressed as IC_{50} (50% inhibitory concentration) defined as the concentration at which the compounds reduce cell proliferation by 50% and are shown in Table 1. As reference compounds we have included colchicine (**1**) and our previous hit compound **4**. Those compounds with a heteroaromatic ring in fragment **A** showed a diverse behavior in terms of anti-proliferative activity. Thus, while the 4-pyridyl derivative (**8a**) had no anti-proliferative activity, the 3-pyridil (**8b**) and 2-pyridyl (**8c**) derivatives were moderately active with IC_{50} values around 1 to 6 μM. The 2-thiophenyl derivative (**8d**) showed better IC_{50} values whereas the 2-furanyl compound (**8e**) afforded IC_{50} values quite comparable to our previous hit compound **4**. Compounds **11a** and **11b** modified in fragment **C** were found to be inactive or almost inactive in the different cell lines.

The first series of modifications at the *ortho* position of the phenyl in fragment **D**, consisting on replacement of the OMe in compound **4** by a CH_2OH, a NH_2 or a $NHCH_3$ (compounds **14**, **18a** and **18b**, respectively) or replacement of the phenyl by a 2-pyridyl (compound **16**), resulted in a very significant drop of the anti-proliferative activity. However, when the OMe in compound **4** was replaced by other alkoxy groups (compounds **20a-d),** a series of potent anti-proliferative compounds was obtained with IC_{50} values in the sub-μM range for the different cell lines tested. The best data were obtained for the 2-ethoxy derivative (**20a**) with IC_{50} values ranging from 0.08 to 0.19 μM against all the cell lines tested. The isopropoxy (**20c**) and the cyclopropylmethoxy (**20d**) derivatives also afforded low IC_{50} values opening the way for exploring other alkoxy substituents. Elongating the alkoxy substituent, such as in compounds with an ethylene glycol (**22a**), propylene glycol (**22b**) or diethylene glycol (**22c**) substituent, kept the anti-proliferative activity in the sub-μM range. When the terminal group at the diethylene glycol chain was replaced by an amino group instead of an OH (compound **22d** versus **22c**), the IC_{50} values were increased 2- to 10-fold. It is noticeable that the introduction of a morpholinoethoxy group (as in **24**) or a piperididyl substituent (as in compound **26**) afforded a significant drop in the cytostatic activity against all cell lines.

Solubility determination.

Experimental solubility determinations were performed in aqueous pH 7.4 buffer for compound **4** and for a selection of the here described compounds with significant antiproliferative activity (**8e**, **20a**, **22b** and **22c**).

Compounds **4** and **20a** exhibited a very poor solubility (s <10 μM). Replacement of the phenyl ring in **A** by a furanyl ring led to a more soluble compound than **4** (**8e**, s=32 ± 0 μM). Also compound **22b,** with a 3-hydroxypropoxy substituent at ring **D** has a better solubility value (s=42 ± 3μM) when compared with **4** or **20a,** with a methoxy or an ethoxy group, respectively. As expected, the best results were obtained with compound **22c** with a diethylene glycol substituent that showed a solubility value of 119 ± 3 μM, more than 10-times more soluble than **4** or **20a.** It should be noted that these solubility values were not correctly predicted using two different computational tools, as can be seen in the Supporting information (Table S1)

Tubulin binding

Compound **4** was previously shown to bind tubulin at the colchicine-binding site.[10] Therefore, the binding of **20a** and **22c** to tubulin was further analyzed by western blot analysis, using EBI, which cross-links the cysteine residues at positions 239 and 354 of β-tubulin, located in the colchicine binding site. This β-tubulin adduct formed by EBI is easily detectable by western blot as a second immunoreactive band that migrates faster than the reference β-tubulin band. As a consequence, treatment of the cells with a compound that binds to this colchicine-binding site will impair the binding of EBI, resulting in the absence of the second band. As shown in Fig. 3, compounds **20a** and **22c** were able to inhibit the formation of the EBI adduct at 40 μM, compound **20a** being equally effective as the previously reported compound **4**, while compound **21c** was less effective.[10]

In order to determine the binding affinities of the here described compounds **8e**, **20a**, **20c** and **22c** for tubulin competition experiments with (R)-(+)-ethyl 5-amino 2-methyl-1,2-dihydro-3-phenylpyrido[3,4-*b*]pyrazin-7-yl carbamate (R-PT), a well characterized reversible colchicine-binding were performed.[20]

The binding constants obtained for compounds **8e**, **20a** and **20c** (Table 2) were similar to the one reported for compound **4** and higher than those previously determined for other classical colchicine-binding site ligands, such as nocodazole (4×10^5 M^{-1}),[21] or podophyillotoxin (1.8×10^6 M^{-1}).[22] It should be emphasized that the Ka value obtained for the 2-ethoxy derivative **20a** was almost identical to that of colchicine. The glycol derivative **22c** had a Ka value of $1.2x10^6$ M^{-1}, closer to that of podophyllotoxin, and ten-fold less potent that our best derivative **20a**. Thus, it can be concluded that these new analogues also bind at the colchicine-binding site in tubulin and compound **20a** has shown an affinity slightly better than our previous best compound **4**.

Docking studies

To gain insight into the molecular basis of the interaction of these compounds with tubulin, a docking study was carried out using the DAMA-colchicine-tubulin complex (Protein Data Bank code: 1SA0)[23] as template. In addition, and since the cyclohexanediones were identified based on the TN-16 binding mode, the TN-16-tubulin complex (PDB ID: 3HKD)[9] was used to expand the grid box in order to cover the so called "colchicine-binding domain". Compound **20a**, having the best affinity constant for tubulin among the here described compounds, was docked using the automated docking program AutoDock 4.0.[24, 25]

The predicted binding mode of **20a** with tubulin (Figure 4) showed only a partial overlap with colchicine (represented in green in Figure 4). Compound **20a** is located deeply into the β-subunit of tubulin, making use of the TN-16 subpocket. Thus, fragment **A** of **20a** is buried inside the β-subunit fitting into a cavity formed by the side chains of residues Valβ238, Leuβ242, Thrβ239,Tyrβ202, Gluβ200 and Asnβ167. The mostly lipophilic nature of this subpocket may help to explain the lack of activity of the pyridine derivatives (**8a-c**) that are probably protonated at physiological pH. It should be noted that the NH at fragment **D** and one of the CO groups of the cyclohexanedione of **20a** are correctly oriented to form an intramolecular hydrogen bond (NH··CO distance 2.2 Å), creating a type of pseudocycle. Such an intramolecular hydrogen bond had already been suggested based on experimental ^{1}H NMR data, and it may be relevant to determine the overall conformation of these compounds making them suitable to bind similarly to TN-16.[10] Indeed, in other colchicine-site binders like the didehydropiperazine diones, a pseudotricycle structure has also been proposed as required for fitting to the colchicine binding site.[26]

In this docked conformation of **20a**, the second carbonyl group of the cyclohexanedione moiety is oriented towards the side chain of residue Cys241, suggesting a hydrogen bond with this residue. Noticeably, DAMA-colchicine and most colchicine-site binders form a hydrogen bond with this cysteine residue.[8] Finally, fragment **D** of compound **20a** is located closer to the αβ-tubulin interface surrounded by residues Leuβ248, Thrβ353, Alaβ354 and Lysβ352 and overlapping with **B**- and **C**-rings of colchicine.

Based on our previous results, only *ortho* substituents at ring **D** have been explored herein. It should be noted that while protic or protonable substituents are not well tolerated (as shown by the poor activity of compounds **14**, **16** o **18a,b**), alkoxy groups, exemplified by the ethoxy group in compound **20a,** result in the most potent compounds. It may be proposed that such alkoxy substituents are directed towards the αβ-tubulin interface, so that the elongation of the substituent (compounds **20b-d)** and the inclusion of a terminal hydroxyl group

(compounds **22a-c)** are compatible with a potent antiproliferative activity. These observations might be helpful for other classes of colchicine-site binders since the highly lipophilic nature of the colchicine site implies that, in most cases, the best ligands have a high lipophilic character for which aqueous solubility becomes a serious problem.

Inhibition of cell cycle progression.

Tubulin-binding agents typically inhibit cell mitosis, leading to inhibition of cell proliferation and/or induction of cell death by apoptosis. Therefore, we investigated the effect of **20a** and **22c** on cell cycle progression. Whereas control endothelial cells show a typical distribution of cells in the different phases of the cell cycle (Figure 5), **20a** caused a dose-dependent increase in the G2/M phase population, indicating that the treated cells can no longer proceed through mitosis. Moreover, **20a** also induced apoptosis, as indicated by the increase in sub G1 cells displaying a sub diploid DNA content, which was particularly evident at 0.3 μM. The glycol derivative **22c** showed a similar effect on cell cycle progression as **20a** with a more pronounced accumulation of cells in G2/M phase at 1 μM, but was less active than **20a** at lower concentrations, which is in agreement with the affinity constant and the EBI-results.

Vascular-disrupting activity.

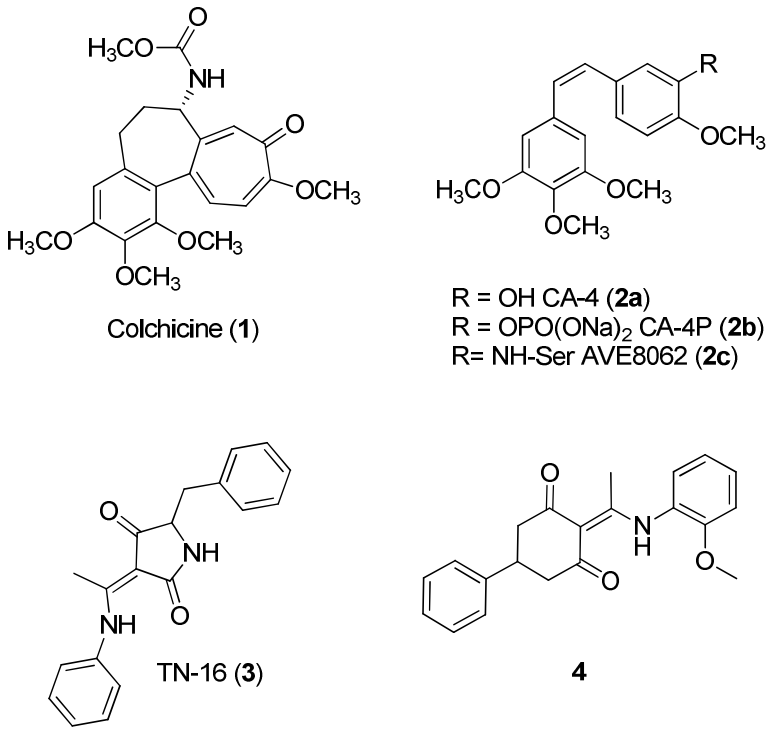
Colchicine-site binding agents have been shown to destroy a preexisting vasculature network formed by endothelial cells and we have seen this effect with compound **4**.[10] Therefore, we tested the vascular-disrupting effect of **20a** and the more soluble analogue **22c**. HMEC-1 cells were seeded on top of matrigel, which induces within 3 h the formation of a network of endothelial cell tubes. Then, the cultures were treated with each compound for 90 min after which the integrity of the vascular network was evaluated. As shown in Figure 6, both compounds displayed a vascular-disrupting activity in a dose-dependent manner, **20a** being about 3-fold more active than **22c**. The images clearly show the absence of a tubular network in cultures treated with 30 and 10 μM of **20a**, whereas at 3 μM a network of vascular structures is still present but several tubes are not connected.

Conclusions

Colchicine-site binders represent a stimulating class of pharmacological compounds due to their antitumoral activity through a combination of antimitotic and antivascular actions. We have recently identified a new

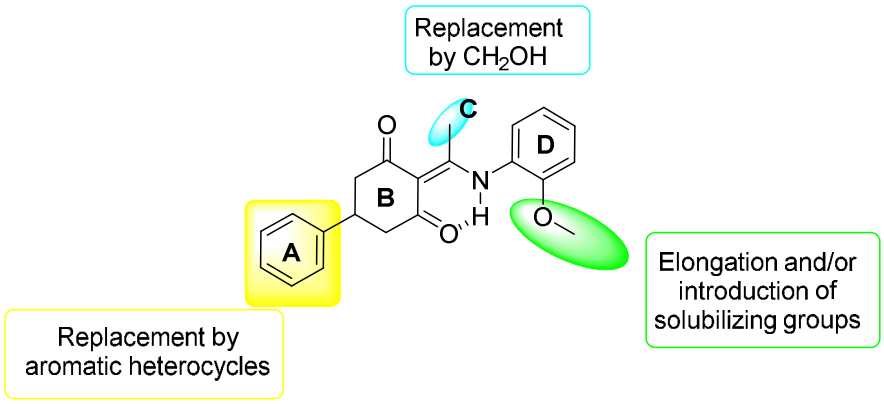
family of colchicine-site binders and herein we have additionally explored structural analogues of our hit named 2-(1-((2-methoxyphenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (**4**). The structure-activity relationship studies indicate that the phenyl ring at position 5 of the cyclohexanedione scaffold (designated as fragment A) can be replaced by a furanyl ring (compound **8e**), keeping a similar binding to colchicine (Ka $4.1x10^6$ M^{-1}), a potent anti-proliferative activity (IC_{50} values ranging from 0.15 to 0.63 μM) and a slightly improved solubility. However replacement by other heteroaromatic rings resulted in significantly less active compounds. On the other hand, among the substituents assayed affecting the *ortho* position in fragment D, the best results were obtained with the alkoxy groups that, according to our docking studies, could be oriented towards the αβ-dimer interface. It should be emphasized that the 2-ethoxy derivative (**20a**) showed an antiproliferative activity in the low μM range (0.08-0.19 μM) and an association constant with tubulin analogous to that of colchicine (Ka $1.3x10^7$ M^{-1}). Incorporation of a diethlyeneglycol substituent facing the dimer interface improved solubility more that 10-fold compared to our previous best compound **4**. Although this increase in aqueous solubility was accompanied by a reduction in affinity in the binding to tubulin and biological activity, this approach may be a valuable tool to increase solubility of other colchicine-site binders.

Figure 1

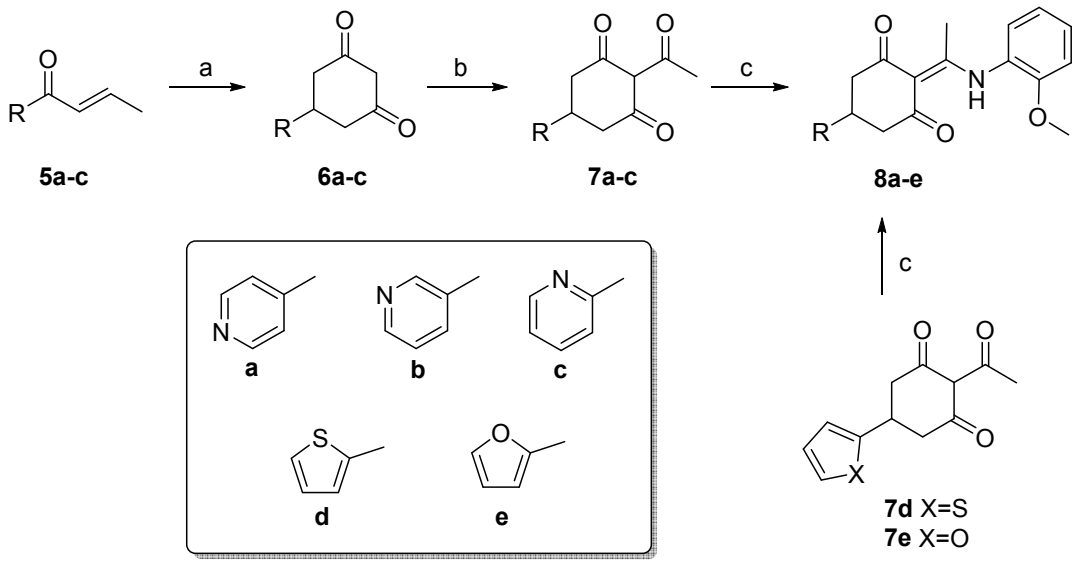


Chemical structures of selected colchicine-site binders

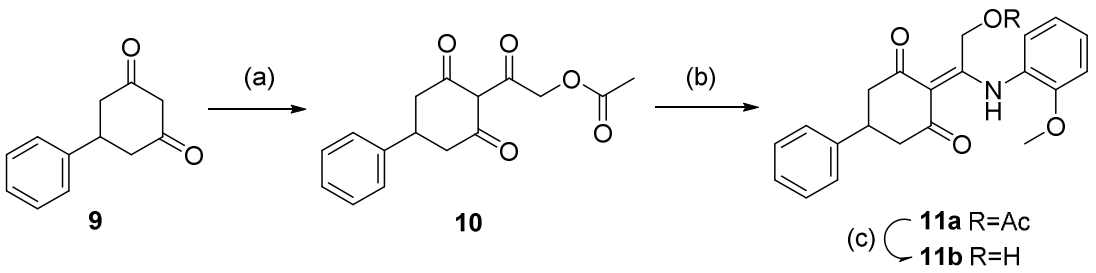
Figure 2



Proposed modifications on **4**

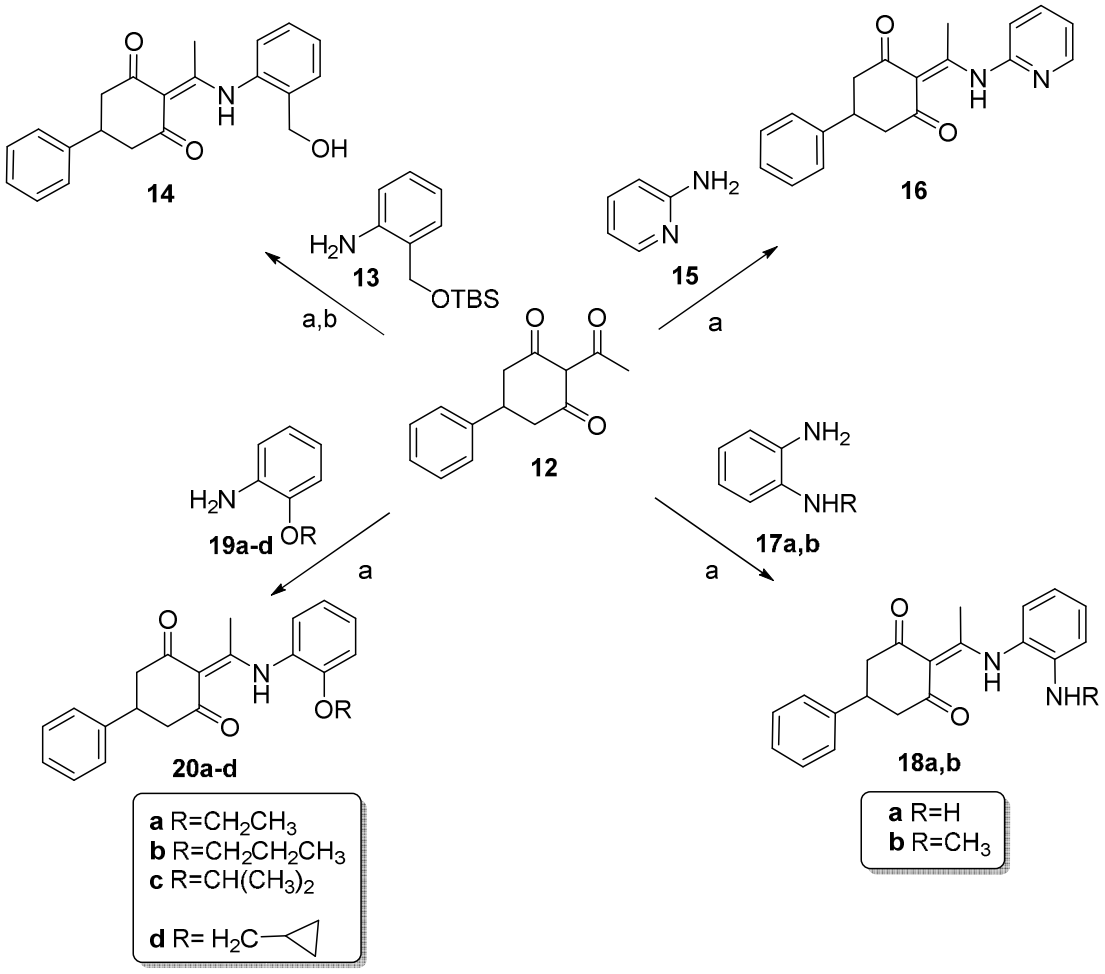


Scheme 1. Reagents and conditions: (a) (i) Diethyl malonate, EtONa, EtOH, Δ; (ii) NaOH, 80 ºC, 2 h; (iii) HCl, Δ, 1 h; (b) $ClCOCH_3$, K_2CO_3 anh, 1,2.4-triazole, Bu_4NBr, DMF, MW, 70 ºC, 2 h; (c) *o*-anisidine, toluene, 4Å molecular sieves, pressure tube, 110 ºC, overnight.

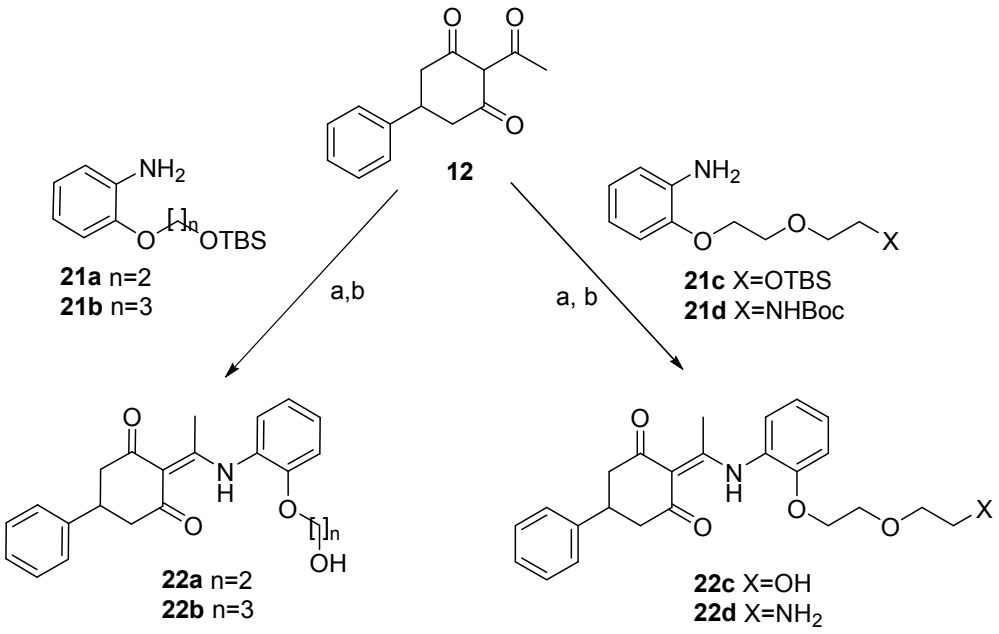


9 10

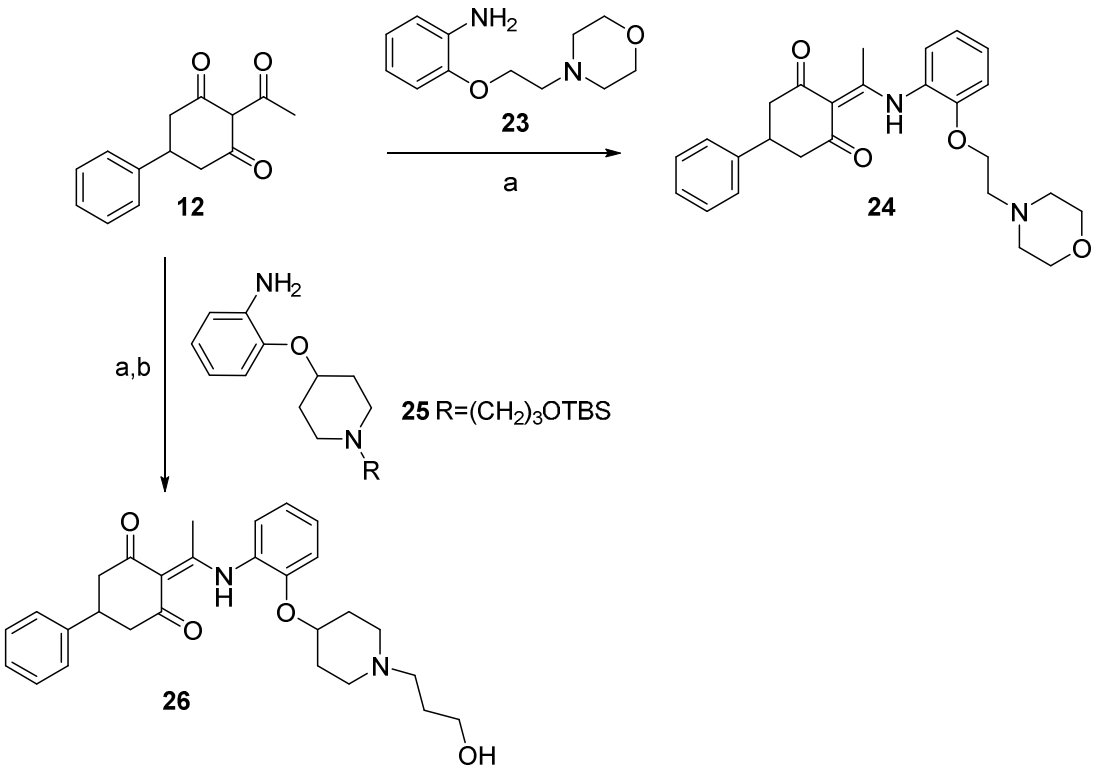
Scheme 2. Reagents and conditions: (a) $ClCOCH_2OCOCH_3$, Et_3N, DMAP, $CHCl_2$, rt, 3h.; (b) *o*-anisidine, toluene, 4Å molecular sieves, pressure tube, 110 ºC, overnight; (c) K_2CO_3, MeOH, rt, 1h.



Scheme 3. Reagents and conditions: (a) Toluene, 4Å molecular sieves, pressure tube, 110 ºC, overnight; (b) TBAF, THF, rt, 1 h.



Scheme 4. Reagents and conditions: (a) Toluene, 4Å molecular sieves, pressure tube, 110 ºC, overnight; (b) TBAF, THF, rt, 1 h; or TFA, $CHCl_3$, rt, overnight (for **22d**)



Scheme 5. Reagents and conditions: (a) Toluene, 4Å molecular sieves, pressure tube, 110 ºC, overnight; (b) TBAF, THF, rt, 1 h

Table 1. Anti-proliferative activity of compounds 8a-e, 11a-b, 14, 16, 18a-b, 20a-d, 22a-d, 24 and 26 in endothelial and tumor cell lines.

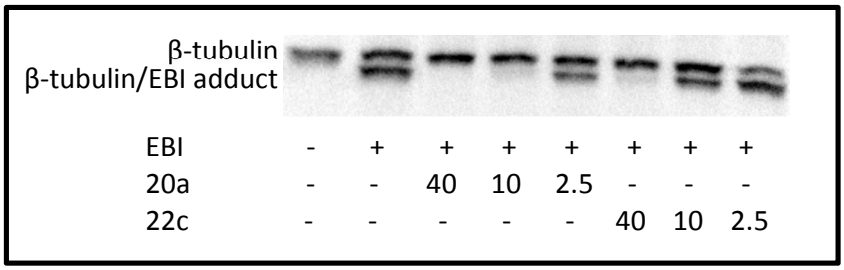
	Endothelial cells IC_{50} (μM)		Tumor cells IC_{50} (μM)		
Compound	HMEC-1	BAEC	L1210	CEM	HeLa
Colchicine	0.0038 ± 0.0011	0.069 ± 0.0008	0.010 ± 0.0006	0.013 ± 0.0004	0.0087 ± 0.0001
4	0.09 ± 0.01	0.09 ± 0.01	0.16 ± 0.08	0.18 ± 0.05	0.18 ± 0.00
8a	>100	>100	>100	>100	>100
8b	3.3 ± 0.5	1.4 ± 0.1	1.8 ± 0.8	4.3 ± 1.0	4.3 ± 1.0
8c	1.7 ± 0.6	1.6 ± 0.2	4.6 ± 0.5	6.5 ± 0.3	5.4 ± 0.4
8d	0.41	0.38	0.59 ± 0.54	0.55 ± 0.41	1.1 ± 0.4
8e	0.24 ± 0.08	0.15 ± 0.04	0.21 ± 0.05	0.34 ± 0.04	0.63 ± 0.37
11a	> 100	> 100	> 250	113 ± 11	≥ 250
11b	48 ± 10	50 ± 14	54 ± 36	33 ± 7	86 ± 1
14	31 ± 1	22 ± 1	102 ± 8	117 ± 29	113 ± 1
16	> 100	58 ± 23	74 ± 17	93 ± 21	112 ± 10
18a	29 ± 2	14 ± 1	14 ± 5	21 ± 5	27 ± 2
18b	14 ± 2	9.4 ± 0.1	12 ± 6	13 ± 9	18 ± 1
20a	0.10 ± 0.02	0.086 ± 0.024	0.19 ± 0.05	0.19 ± 0.01	0.18 ± 0.00
20b	0.48 ± 0.02	0.35 ± 0.11	0.71 ± 0.04	0.86 ± 0.10	0.81 ± 0.11
20c	0.16 ± 0.07	0.12 ± 0.09	0.23 ± 0.03	0.19 ± 0.02	0.32 ± 0.04
20d	0.24 ± 0.02	0.23 ± 0.09	0.25 ± 0.03	0.22 ± 0.06	0.62 ± 0.06
22a	0.83 ± 0.03	0.69 ± 0.12	0.85 ± 0.28	1.1 ± 0.4	2.2 ± 1.6
22b	0.50 ± 0.06	0.53 ± 0.03	0.87 ± 0.09	0.82 ± 0.02	1.1 ± 0.
22c	0.46 ± 0.05	0.45 ± 0.03	0.80 ± 0.01	0.42 ± 0.14	0.93 ± 0.05
22d	1.5 ± 0.3	2.7 ± 2.5	1.2 ± 0.0	4.3 ± 1.3	1.5 ± 0.0
24	13 ± 1	22 ± 1	28 ± 4	25 ± 1	54 ± 36
26	21 ± 5	30 ± 6	19 ± 3	26 ± 4	57 ± 39

Table 2. Association constants for compounds 4, 8e, 20a, 20c and 22c, and other colchicine-binding site ligands

Compound	K_{assoc} (M^{-1}) 25ºC
Colchicine	1.16 x10^7 (at 37ºC)[a]
R-PT	3.2 x 10^{6} [b]
Podophyillotoxin	1.8 x 10^{6} [c]
Nocodazole	4 x 10^{5} [d]
4	(9.6 ± 1.2) x 10^6
8e	(4.1 ± 0.3) x10^6
20a	(1.3 ± 0.2) x10^7
20c	(6.3 ± 0.8) x10^6
22c	(1.2 ± 0.1) x10^6

[a]Data from ref. 27. [b]Data from ref. 28 [c]Data from ref. 22. [d]Data from ref. 21

Figure 3



Tubulin binding. MDA-MB-231 cells were treated with DMSO, **20a** or **22c** at 40, 10 or 2.5 μM for 24 h. Next EBI (100 μM) was added and after 1.5 h, the cells were harvested and cell extracts were prepared for western blot analysis using anti-β-tubulin antibody. EBI cross-links cysteine residues in β-tubulin resulting in the formation of a β-tubulin/EBI adduct (second immunoreactive band). Compounds that bind to the colchicine-binding site in β-tubulin prevent the formation of the EBI/β-tubulin adduct

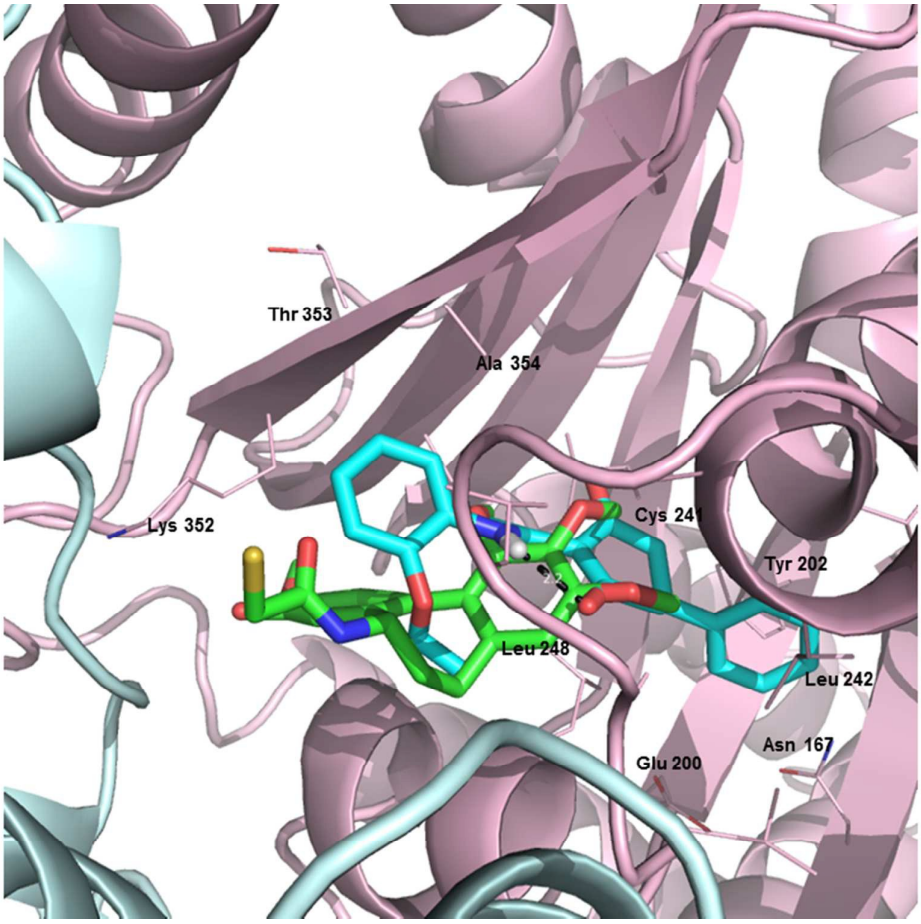


Figure 4. Predicted binding mode for **20a** (cyan) in the colchicine binding domain of αβ-tubulin (α-tubulin in pale cyan and β-tubulin in light pink), and overlap with colchicine (green). Dashed lines indicate hydrogen bonds. Surrounding amino acid side chains of residues in the binding site within 4 Å from **20a** are shown in lines and labelled.

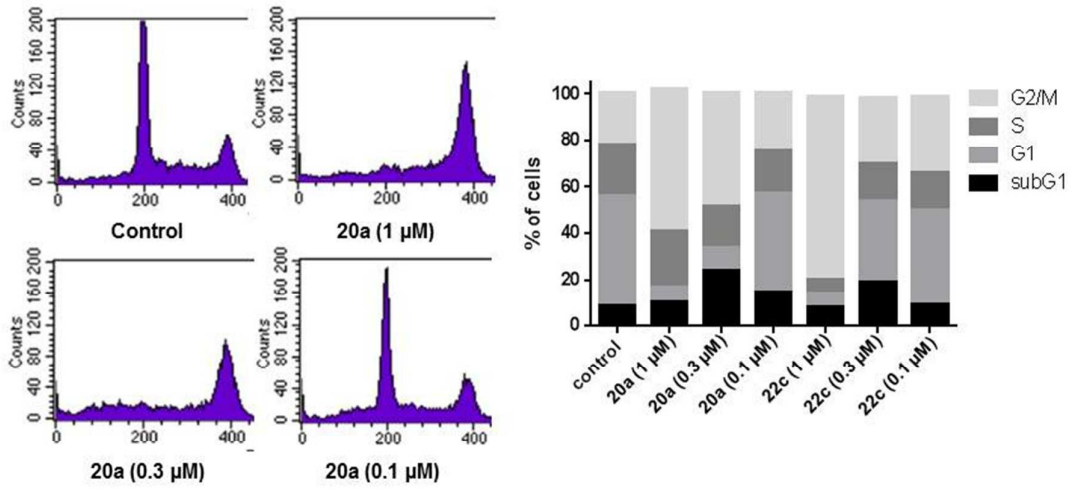


Figure 5. **Inhibition of cell cycle progression.** HMEC-1 were treated with DMSO (control) or different concentrations of **20a** or **22c** for 24 h. for 24 h . Next, the cells were harvested, stained with propidium iodide, and cell cycle distribution was evaluated by flow cytometry. Percentages of cells in the different phases of the cell cycle are indicated.

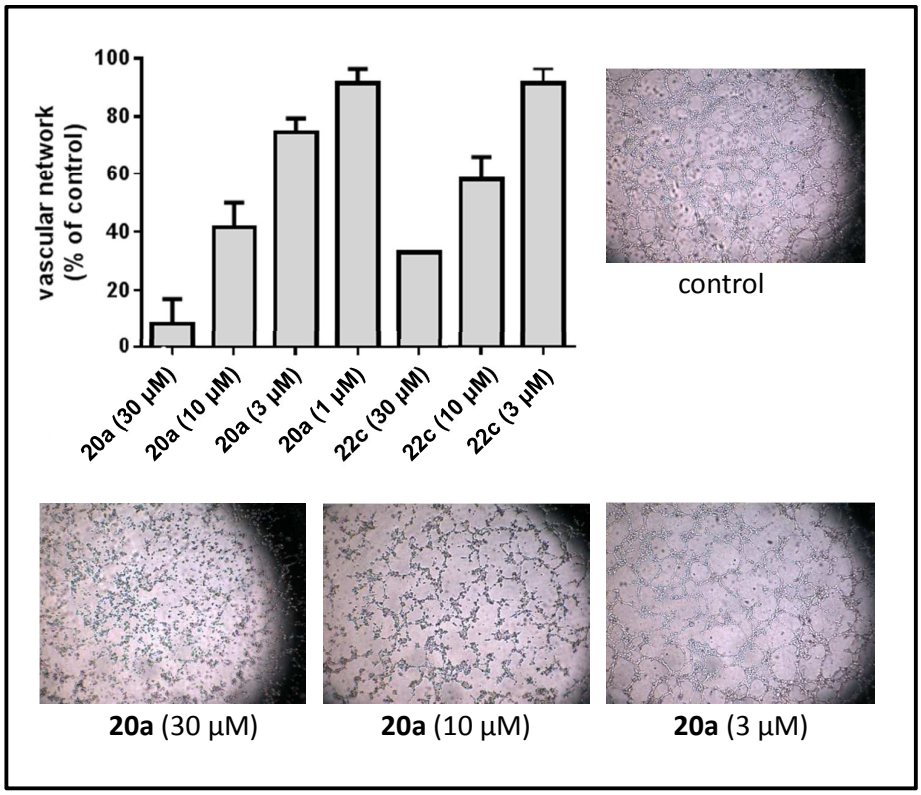


Figure 6. **Vascular disrupting effects of 20a and 22c**. HMEC-1 cells were cultured on matrigel for 3 h to allow the formation of tube-like structures. Then different concentrations of compounds were added. After 90 min, tube formation was quantified. Values are expressed as mean ± SD. Images show the disruption of the vascular network after treatment with **20a**.

Experimental Section.

Chemistry procedures

Melting points were obtained on a Reichert-Jung Kofler apparatus and are uncorrected. The elemental analysis was performed with a Heraeus CHN-O-RAPID instrument. The elemental compositions of the compounds agreed to within ±0.4% of the calculated values. For all the tested compounds, satisfactory elemental analysis was obtained supporting >95% purity. Electrospray mass spectra were measured on a quadrupole mass spectrometer equipped with an electrospray source (Hewlett-Packard, LC/MS HP 1100). ^{1}H and ^{13}C NMR spectra were recorded on a Varian INNOVA 300 operating at 299 MHz (^{1}H) and 75 MHz (^{13}C), respectively, a Varian INNOVA-400 operating at 399 MHZ (^{1}H) and 99 MHz (^{13}C), respectively, and a VARIAN SYSTEM-500 operating a 499 MHz (^{1}H) and 125 MHz (^{13}C), respectively.

Analytical TLC was performed on silica gel 60 F_{254} (Merck) precoated plates (0.2 mm). Spots were detected under UV light (254 nm) and/or charring with ninhydrin or phosphomolibdic acid. Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a Chromatotron^{R} (Kiesegel 60 PF_{254} gipshaltig (Merck)), with layer thickness of 1 and 2 mm and flow rate of 4 or 8 mL/min, respectively. Flash column chromatography was performed in a Biotage Horizon instrument.

Microwave reactions were performed using the Biotage Initiator 2.0 single-mode cavity instrument from Biotage (Uppsala). Experiments were carried out in sealed microwave process vials utilizing the standard absorbance level (400 W maximum power). The temperature was measured with an IR sensor on the outside of the reaction vessel.

5-(Pyridin-4-yl)cyclohexane-1,3-dione (6a).To a solution of 25% sodium ethoxide in ethanol (3.70 mL, 13.55 mmol) diethyl malonate (2.06 mL, 13.55 mmol) was added dropwise while keeping the temperature below 25 ºC. The mixture was diluted with ethanol (0.2 mL) and heated at 60 ºC. Once the temperature was reached, a solution of (*E*)-4-(pyridin-4-yl)but-3-en-2-one[29] (**5a**) (1.81 g, 12.32 mmol) in ethanol (3 mL) was added and the reaction was refluxed. The course of the reaction was monitored by LC-MS until the corresponding starting material was consumed. Then a solution of 6 M sodium hydroxide (0.3 mmol) was added and the reaction was heated at 80 ºC for 2h. After cooling, ethanol was removed in vacuo and the resulting solution was washed with toluene (2x10 mL). The aqueous layer was treated with 37% HCl until pH 2, refluxed for 1h and left to cool at rt. Then, the mixture was neutralized with 6M sodium hydroxide and extracted with isobutanol:ethyl acetate (1:1) (3x20 mL). The organic extracts were dried on anhydrous

Na_2SO_4, filtered and evaporated, which afforded **6a** (1.42 g, 61%) as a yellow oil, pure enough for the next step. MS (ES, positive mode): m/z 190 $(M+H)^+$. ^1H NMR (DMSO-d_6, 300 MHz) δ (enol form): 2.40 (dd, 2H, *J* = 16.7, 4.6 Hz, H-4, H-6), 2.59 (m, 2H, H-4, H-6), 3.34 (m, 1H, H-5), 5.23 (s, 1H, H-2), 7.36 (m, 2H, *J* = 4.2, 1.6 Hz, Ar), 8.50 (m, 2H, *J* = 4.6, 1.4 Hz, Ar).

5-(Pyridin-3-yl)cyclohexane-1,3-dione (6b). As described for the synthesis of **6a**, a mixture of diethyl malonate (1.36 mL, 8.97 mmol), 25% sodium ethoxide in ethanol (2.44 mL, 8.97 mmol) and (*E*)-4-(pyridin-3-yl)but-3-en-2-one[29] (**5b**) (1.20 g, 8.15 mmol) afforded **6b** (0.77 g, 50%) as a yellow oil, pure enough for the next step. MS (ES, positive mode): m/z 190 $(M+H)^+$. ^1H NMR (DMSO-d_6, 300 MHz) δ (enol form): 2.10 (dd, 2H, *J* = 15.7, 4.5 Hz, H-4, H-6), 2.23 (m, 2H, H-4, H-6), 3.15 (tt, 1H, *J* = 11.4, 4.7 Hz, H-5), 4.51 (s, 1H, H-2), 7.28 (m, 1H, Ar), 7.66 (dt, 1H, *J* = 7.8, 2.1 Hz, Ar), 8.37 (dd, 1H, *J* = 4.7, 1.7 Hz, Ar), 8.47 (d, 1H, *J* = 2.4 Hz, Ar).

5-(Pyridin-2-yl)cyclohexane-1,3-dione (6c). As described for the synthesis of **6a**, a mixture of diethyl malonate (1.36 mL, 8.97 mmol), 25% sodium ethoxide in ethanol (2.44 mL, 8.97 mmol) and (*E*)-4-(pyridin-2-yl)but-3-en-2-one[29] (**5c**) (1.20 g, 8.15 mmol) yielded **6c** (1.51 g, 97%) as a yellow oil, that was used as such for the next step. MS (ES, positive mode): m/z 190 $(M+H)^+$. ^1H NMR (DMSO-d_6, 300 MHz) δ (enol form): 2.08 (m, 2H, *J* = 16.3, 4.5 Hz, H-4, H-6), 2.32 (m, 2H, H-4, H-6), 3.23 (tt, 1H, *J* = 11.7, 4.7 Hz, H-5), 4.51 (s, 1H, H-2), 7.17 (ddd, 1H, *J* = 7.4, 4.8, 1.2 Hz, Ar), 7.27 (dt, 1H, *J* = 7.8, 0.9 Hz, Ar), 7.67 (td, 1H, *J* = 7.6, 1.9 Hz, Ar), 8.47 (m, 1H, Ar).

2-Acetyl-5-(pyridin-4-yl)cyclohexane-1,3-dione (7a). A microwave vial was charged with **6a** (375 mg, 1.98 mmol), acetylchloride (305 µL, 3.96 mmol), anhydrous K_2CO_3 (603 mg, 4.36 mmol), 1,2,4-triazole (55 mg, 0.79 mmol) and tetrabutylammonium bromide (319 mg, 0.99 mmol) in anhydrous DMF (8 mL). The reaction vessel was sealed, stirred under argon atmosphere for 10 minutes and heated in a microwave reactor at 70 ºC for 2 h. After cooling, the reaction mixture was acidified with 1N HCl and the crude was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4, concentrated, and purified by flash chromatography (hexane/ethyl acetate) to yield **7a** (170 mg, 37%) as a white solid. Mp 121-123 ºC. MS (ES, positive mode): 232 m/z $(M+H)^+$. ^1H NMR (DMSO-d_6, 300 MHz) δ (enol form): 2.55 (s, 3H, CH_3), 2.76 (m, 2H, H-4, H-6), 2.95 (m, 2H, H-4, H-6), 3.46 (tt, 1H, *J* = 11.7, 4.2 Hz, H-5), 7.37 (m, 2H, *J* = 4.4, 1.8 Hz, Ar), 8.52 (m, 2H, *J* = 4.3, 1.8 Hz, Ar).

2-Acetyl-5-(pyridin-3-yl)cyclohexane-1,3-dione (7b). As described for the synthesis of **7a**, a microwave vial was charged with **6b** (334 mg, 1.76 mmol), acetylchloride (249 µL, 3.52 mmol), anhydrous K_2CO_3 (535 mg,

3.87 mmol), 1,2,4-triazole (48 mg, 0.70 mmol) and tetrabutylammonium bromide (284 mg, 0.88 mmol) in anhydrous DMF (7 mL). After workup and flash chromatography (hexane/ethyl acetate) **7b** (151 mg, 37%) was obtained as an oil. MS (ES, positive mode): 232 m/z $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (enol form): 2.56 (s, 3H, CH_3), 2.73 (m, 2H, H-4, H-6), 3.01 (m, 2H, H-4, H-6), 3.48 (tt, 1H, *J* = 12.0, 4.1 Hz, H-5), 7.38 (ddd, 1H, *J* = 7.7, 4.8, 0.9 Hz, Ar), 7.78 (dt, 1H, *J* = 8.0, 2.0 Hz, Ar), 8.47 (dd, 1H, *J* = 4.7, 1.7 Hz, Ar), 8.56 (d, 1H, *J* = 2.1 Hz, Ar).

2-Acetyl-5-(pyridin-2-yl)cyclohexane-1,3-dione (7c). As described for the synthesis of **7a**, a microwave vial was charged with **6c** (334 mg, 1.76 mmol), acetylchloride (249 µL, 3.52 mmol), anhydrous K_2CO_3 (535 mg, 3.87 mmol), 1,2,4-triazole (48 mg, 0.70 mmol) and tetrabutylammonium bromide (284 mg, 0.88 mmol) in anhydrous DMF (7 mL) to yield **7c** (200 mg, 49%) as white solid. Mp 91-93 ºC. MS (ES, positive mode): 232 m/z $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (enol form): 2.52 (s, 3H, CH_3), 2.83 (m, 2H, H-4, H-6), 3.03 (m, 2H, H-4, H-6), 3.60 (tt, 1H, *J* = 9.9, 4.7 Hz, H-5), 7.27 (ddd, 1H, *J* = 7.4, 4.8, 1.1 Hz, Ar), 7.39 (dt, 1H, *J* = 7.8, 1.0 Hz, Ar), 7.77 (td, 1H, *J* = 7.7, 1.8 Hz, Ar), 8.52 (ddd, 1H, *J* = 4.9, 1.9, 0.9, Ar), 11.98 (br s, 1H, OH).

General procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines. A solution of the corresponding 2-acylcyclohexane-1,3-dione (1.0 mmol) and the appropriate aniline (1.5 mmol) in toluene (10 mL) was placed in an Ace pressure tube. Then, 4 Å molecular sieves were added, the vessel was sealed and heated at 110 ºC overnight. After cooling, the solvent was evaporated and the residue was purified by flash chromatography (hexane/ethyl acetate).

2-(1-((2-Methoxyphenyl)amino)ethylidene)-5-(pyridin-4-yl)cyclohexane-1,3-dione (8a). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of **7a** (150 mg, 0.65 mmol) and *o*-anisidine (110 µL, 0.97 mmol) in toluene afforded **8a** (96 mg, 44%) as a white solid. Mp 146-148 ºC. MS (ES, positive mode): m/z 337 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.41 (s, 3H, CH_3), 2.64 (m, 2H, H-4, H-6), 2.83 (m, 2H, H-4, H-6), 3.39 (tt, 1H, *J* = 11.5, 3.8 Hz, H-5), 3.83 (s, 3H, OCH_3), 7.04 (td, 1H, *J* = 7.6, 1.2 Hz, Ar), 7.20 (dd, 1H, *J* = 8.4, 1.2 Hz, Ar), 7.32 (dd, 1H, *J* = 7.8, 1.5 Hz, Ar), 7.37 (m, 2H, Ar), 7.40 (m, 1H, Ar), 8.51 (d, 1H, *J* =1.6 Hz, Ar), 8.51 (d, 1H, *J* = 1.6 Hz, Ar), 14.75 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 19.8 (CH_3), 35.3 (C-5), 45.1 (C-4, C-6), 55.8 (OCH_3), 108.4 (NHC=C), 112.3, 120.6, 122.3, 124.4, 126.9, 129.2, 149.7, 152.0, 153.0 (Ar), 172.6 (NHC=C). Anal. calc. for ($C_{20}H_{20}N_2O_3$): C, 71.41; H, 5.99; N, 8.33. Found: C, 71.70; H, 6.18; N, 8.10.

2-(1-((2-Methoxyphenyl)amino)ethylidene)-5-(pyridin-3-yl)cyclohexane-1,3-dione (8b). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of **7b** (130 mg, 0.56 mmol) and *o*-anisidine (95 μL, 0.84 mmol) in toluene yielded **8b** (105 mg, 56%) as an oil. MS (ES, positive mode): m/z 337 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 300 MHz) (DMSO-d_6, 300 MHz) δ: 2.41 (s, 3H, CH_3), 2.63 (dd, 2H, *J* = 15.6, 3.1 Hz, H-4, H-6), 2.87 (m, 2H, H-4, H-6), 3.39 (m, 1H, H-5), 3.84 (s, 3H, OCH_3), 7.04 (td, 1H, *J* = 7.6, 1.1 Hz, Ar), 7.20 (dd, 1H, *J* = 8.4, 0.8 Hz, Ar), 7.32 (m, 1H, Ar), 7.37 (m, 2H, Ar), 7.78 (dt, 1H, *J* = 7.9, 1.9 Hz, Ar), 8.46 (dd, 1H, *J* = 4.7, 1.5 Hz, Ar), 8.57 (d, 1H, *J* = 2.0 Hz, Ar), 14.76 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 19.7 (CH_3), 33.7 (C-5), 45.1 (C-4, C-6), 55.8 (OCH_3), 108.4 (NHC=C), 112.3, 120.6, 123.5, 124.4, 126.9, 129.2, 134.3, 138.8, 147.8, 148.6, 153.1 (Ar), 172.6 (NHC=C). Anal. calc. for ($C_{20}H_{20}N_2O_3$): C, 71.41; H, 5.99; N, 8.33. Found: C, 71.19; H, 6.02; N, 8.12.

2-(1-((2-Methoxyphenyl)amino)ethylidene)-5-(pyridin-2-yl)cyclohexane-1,3-dione (8c). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of **7c** (180 mg, 0.78 mmol) and *o*-anisidine (132 μL mg, 1.17 mmol) in toluene, afforded **8c** (185 mg, 71%) as a white solid. Mp 127-129 ºC. MS (ES, positive mode): m/z 337 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.40 (s, 3H, CH_3), 2.69 (m, 2H, H-4, H-6), 2.87 (m, 2H, H-4, H-6), 3.50 (tt, 1H, *J* = 11.4, 4.2 Hz, H-5), 3.83 (s, 3H, OCH_3), 7.04 (td, 1H, *J* = 7.6, 1.3 Hz, Ar), 7.21 (dd, 1H, *J* = 8.3, 0.6 Hz, Ar), 7.26 (ddd, 1H, *J* = 7.3, 4.9, 1.1 Hz, Ar), 7.31 (dd, 1H, *J* = 7.9, 1.6 Hz, Ar), 7.37 (m, 2H, Ar), 7.76 (td, 1H, *J* = 7.7, 1.9 Hz, Ar), 8.54 (m, 1H, Ar), 14.77 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 19.7 (CH_3), 37.9 (C-5), 44.3 (C-4, C-6), 55.8 (OCH_3), 108.5 (NHC=C), 112.3, 120.6, 121.8, 121.9, 124.5, 126.9, 129.2, 136.8, 149.0, 153.1, 161.7 (Ar), 172.6 (NHC=C). Anal. calc. for ($C_{20}H_{20}N_2O_3$): C, 71.41; H, 5.99; N, 8.33. Found: C, 71.80; H, 6.21; N, 8.23.

2-(1-((2-Methoxyphenyl)amino)ethylidene)-5-(thiophen-2-yl)cyclohexane-1,3-dione (8d). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of 2-acetyl-5-(thiophen-2-yl)cyclohexane-1,3-dione (**7d**)[11] (60 mg, 0.25 mmol) and *o*-anisidine (43 μL, 0.38 mmol) in toluene afforded **8d (**86 mg, 99%) as a yellow solid. Mp 105-107 ºC. MS (ES, positive mode): m/z 342 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 2.39 (s, 3H, CH_3), 2.80 (m, 4H, H-4, H-6), 3.64 (tt, 1H, *J* = 9.4, 4.6 Hz, H-5), 3.83 (s, 3H, OCH_3), 6.96 (dt, 1H, *J* = 3.5, 1.2 Hz, Ar), 6.98 (dd, 1H, *J* = 5.0, 3.5 Hz, Ar) 7.03 (td, 1H, *J* = 7.6, 1.2 Hz), 7.19 (dd, 1H, *J* = 8.4, 1.1 Hz, Ar), 7.31 (dd, 1H, *J* = 7.8, 1.5 Hz, Ar), 7.38 (m, 2H, Ar), 14.74 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 19.8 (CH_3), 31.6 (C-5), 46.2 (C-4, C-6), 55.9 (OCH_3), 108.6 (NHC=C), 112.3, 120.6, 123.5, 123.9, 124.4, 126.9, 126.9, 129.2, 147.2, 153.1 (Ar), 172.5

(NHC=C). Anal. calc. for ($C_{19}H_{19}NO_3S$): C, 66.84; H, 5.61; N, 4.10; S, 9.39. Found: C, 67.02; H, 5.50; N, 3.89; S, 9.16.

5-(Furan-2-yl)-2-(1-((2-methoxyphenyl)amino)ethylidene)cyclohexane-1,3-dione (8e). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of 2-acetyl-5-(furan-2-yl)cyclohexane-1,3-dione (**7e**)[11] (60 mg, 0.27 mmol) and *o*-anisidine (46 µL, 0.41 mmol) in toluene afforded **8e** (87 mg, 99%) as a yellow solid. Mp 98-100 ºC. MS (ES, positive mode): m/z 326 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 2.38 (s, 3H, CH_3), 2.76 (m, 4H, H-4, H-6), 3.44 (m, 1H, H-5), 3.82 (s, 3H, OCH_3), 6.16 (d, 1H, *J* = 3.2 Hz, Ar), 6.38 (dd, 1H, *J* = 3.2, 1.9 Hz, Ar), 7.03 (td, 1H, *J* = 7.7, 1.0 Hz, Ar), 7.19 (dd, 1H, *J* = 8.3, 0.8 Hz, Ar), 7.30 (dd, 1H, *J* = 7.7, 1.2 Hz, Ar), 7.38 (m, 1H, Ar), 7.57 (d, 1H, *J* = 1.1 Hz, Ar), 14.73 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 19.8 (CH_3), 29.9 (C-5), 42.8 (C-4, C-6), 55.8 (OCH_3), 108.6 (NHC=C), 104.7, 110.4, 112.3, 120.6, 124.4, 126.9, 129.2, 141.8, 153.1, 156.4 (Ar), 172.5 (NHC=C). Anal. calc. for ($C_{19}H_{19}NO_4$): C, 70.14; H, 5.89; N, 4.31. Found: C, 69.95; H, 5.90; N, 4.17.

2,6-Dioxo-4-phenylcyclohexyl)methyl acetate (10) To a solution of 5-phenyl-1,3-cyclohexanedione (**9**) (1.0 g, 5.32 mmol) in dichloromethane (14 mL), triethylamine (1.5 mL, 10.64 mmol) and DMAP (7 mg, 0.06 mmol) were added. Then, acetoxyacetyl chloride (0.62 mL, 5.84 mmol) was added dropwise. After stirring for 3 h at room temperature, acetic acid (0.4 mL, 6.92 mmol) was added and the reaction was further stirred for 30 min. The reaction was diluted with water (10 mL) and extracted with dichloromethane (2x10 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The crude was purified by flash chromatography (hexane/ethyl acetate) to yield **10** (1.04 g, 68%) as a white solid. Mp 116-118 ºC. MS (ES, positive mode): m/z 289 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 2.12 (s, 3H, CH_3), 2.70 (m, 2H, H-4, H-6), 2.98 (m, 2H, H-4, H-6), 3.45 (tt, 1H, *J* = 11.8, 4.2 Hz, H-5), 4.85 (s, 1H, H-2), 5.21 (s, 2H, CH_2), 7.27 (m, 1H, Ar), 7.35 (m, 4H, Ar).

2-(2,6-Dioxo-4-phenylcyclohexylidene)-2-((2-methoxyphenyl)amino)ethyl acetate (11a). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of **10** (1.0 g, 3.47 mmol) and *o*-anisidine (0.59 mL, 5.20 mmol) in toluene afforded **11a (**910 mg, 67%) as an oil. MS (ES, positive mode): m/z 394 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 1.69 (s, 3H, CH_3), 2.64 (m, 2H, H-4, H-6), 2.86 (m, 2H, H-4, H-6), 3.39 (tt, 1H, *J* = 11.9, 3.8 Hz, H-5), 3.84 (s,

3H, OCH_3), 5.22 (s, 2H, CH_2), 7.02 (td, 1H, *J* = 7.6, 1.2 Hz, Ar), 7.18 (dd, 1H, *J* = 8.3, 1.2 Hz, Ar), 7.24 (m, 1H, Ar), 7.30 (dd, 1H, *J* = 7.8, 1.6 Hz, Ar), 7.34 (m, 2H, Ar), 7.35 (m, 2H, Ar), 7.35 (s, 1H, Ar), 14.87 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 19.8 (CH_3), 36.0 (C-5), 46.1 (C-4, C-6), 55.9 (OCH_3), 60.4 (CH_2), 108.3 (NHC=C), 112.3, 120.6, 125.3, 125.6, 126.6, 126.8, 128.5, 129.2, 143.3, 152.6 (Ar), 167.4 (NHC=C), 169.2 ($COCH_3$). Anal. calc. for ($C_{23}H_{23}NO_5$): C, 70.21; H, 5.89; N, 3.56. Found: C, 69.92; H, 6.01; N, 3.68.

2-(2-Hydroxy-1-((2-methoxyphenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (11b). To a solution of **11a** (98 mg, 0.25 mmol) in methanol (1 mL), K_2CO_3 (52 mg, 0.38 mmol) was added and the reaction was stirred at room temperature for 1 h. Then, the reaction was neutralized with 1 N HCl and extracted with ethyl acetate (3x 10 mL). The organic layer was dried over Na_2SO_4, concentrated and purified by flash chromatography (hexane/ethyl acetate) to yield **11b (**60 mg, 68%) as a rosaceus solid. Mp 131-133 ºC. MS (ES, positive mode): m/z 352 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.66 (m, 2H, H-4, H-6), 2.86 (m, 2H, H-4, H-6), 3.35 (tt, 1H, *J* = 12.8, 4.0 Hz, H-5), 3.83 (s, 3H, OCH_3), 4.37 (d, 2H, *J* = 7.2 Hz, CH_2), 5.34 (t, 1H, *J* = 7.2 Hz, OH), 7.04 (t, 1H, *J* = 7.4 Hz, Ar), 7.18 (d, 1H, *J* = 8.0 Hz, Ar), 7.23 (m, 1H, Ar), 7.32 (m, 2H, Ar), 7.33 (m, 2H, Ar), 7.37 (m, 1H, Ar), 7.44 (dd, 1H, *J* = 7.8, 1.1 Hz, Ar), 14.83 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 36.5 (C-5), 46.0 (C-4, C-6), 56.4 (CH_2OH), 58.4 (OCH_3), 108.9 (NHC=C), 112.6, 121.0, 125.2, 126.3, 127.0, 127.2, 129.0, 129.5, 143.7, 152.9 (Ar), 171.2 (NHC=C). Anal. calc. for ($C_{21}H_{22}NO_4$): C, 71.78; H, 6.02; N, 3.99. Found: C, 71.72; H, 5.98; N, 4.01.

2-(1-((2-(Hydroxymethyl)phenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (14). A solution of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (100 mg, 0.43 mmol) and 2-(((*tert*-butyldimetylsily)oxy)methyl)aniline (**13**)[12, 15] (155 mg, 0.65 mmol) in toluene (4 mL) was placed in an Ace pressure tube. Then, 4 Å molecular sieves were added and the vessel was sealed and heated at 110 ºC overnight. After cooling, the solvent was evaporated to dryness and the crude reaction mixture was then dissolved in anhydrous THF (2 mL) and treated with 1M TBAF in THF (400 µL, 0.40 mmol) at room temperature for 1h. The reaction was quenched with water and extracted with dichloromethane (2 x 20 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The crude was purified by flash chromatography (hexane/ethyl acetate) to yield **14** (106 mg, 73% for the two steps) as a white solid. Mp 166-168 ºC. MS (ES, positive mode): m/z 336 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.37 (s, 3H, CH_3), 2.62 (m, 2H, H-4, H-6), 2.83 (m, 2H, H-4, H-6), 3.34 (tt, 1H, *J* = 12.2, 4.0 Hz, H-5), 4.41 (d, 2H, *J* = 5.2 Hz, CH_2),

5.27 (t, 1H, *J* = 5.2 Hz, OH), 7.24 (m, 1H, Ar), 7.28 (dd, 1H, *J* = 7.2, 1.7 Hz, Ar), 7.34 (m, 2H, Ar), 7.35 (m, 2H, Ar), 7.40 (m, 2H, Ar), 7.55 (dd, 1H, *J* = 7.3, 2.0 Hz, Ar), 14.83 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 19.8 (CH_3), 36.1 (C-5), 45.9 (C-4, C-6), 59.5 (CH_2), 108.3 (NHC=C), 126.5, 126.7, 127.9, 128.0, 128.5, 128.6, 134.2, 137.4, 143.5 (Ar), 172.6 (NHC=C). Anal. calc. for ($C_{21}H_{21}NO_3$): C, 75.20; H, 6.31; N, 4.18. Found: C, 75.35; H, 6.60; N, 4.07.

5-Phenyl-2-(1-(pyridin-2-ylamino)ethylidene)cyclohexane-1,3-dione (16). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, reaction of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (100 mg, 0.43 mmol) and 2-aminopyridine (**15**) (62 mg, 0.65 mmol) in toluene afforded **16** (111 mg, 84%) as an oil. MS (ES, positive mode): m/z 307 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.64 (m, 2H, H-4, H-6), 2.68 (s, 3H, CH_3), 2.85 (m, 2H, H-4, H-6), 3.29 (m, 1H, H-5), 7.24 (m, 1H, Ar), 7.34 (d, 4H, *J* = 4.3 Hz, Ar), 7.38 (dd, 1H, *J* = 7.3, 5.0 Hz, Ar), 7.44 (d, 1H, *J* = 8.1 Hz, Ar), 7.95 (td, 1H, *J* = 7.8, 1.7 Hz, Ar), 8.53 (m, 1H, Ar), 15.19 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 20.2 (CH_3), 35.8 (C-5), 46.0 (C-4, C-6), 109.3 (NHC=C), 119.3, 122.4, 126.6, 126.8, 128.5, 139.0, 143.3, 149.1, 150.0 (Ar), 171.0 (NHC=C). Anal. calc. for ($C_{19}H_{18}N_2O_2$): C, 74.49; H, 5.92; N, 9.14. Found: C, 74.27; H, 5.81; N, 9.00.

2-(1-((2-Aminophenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (18a). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, reaction of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (40 mg, 0.17 mmol) and *o*-phenylendiamine (**17a**) (19 mg, 0.17 mmol) in toluene yielded **18a (**45 mg, 83%) as a yellow solid. Mp 135-136 ºC. MS (ES, positive mode): m/z 321 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.37 (s, 3H, CH_3), 2.61 (m, 2H, H-4, H-6), 2.79 (m, 2H, H-4, H-6), 3.31 (m, 1H, H-5), 5.19 (br s, 2H, NH_2), 6.61 (td, 1H, *J* = 7.7, 1.2 Hz, Ar), 6.81 (dd, 1H, *J* = 7.8, 1.0 Hz, Ar), 6.97 (d, 1H, *J* = 7.6 Hz, Ar), 7.09 (t, 1H, *J* = 7.7 Hz, Ar), 7.24 (m, 1H, Ar), 7.34 (m, 2H, Ar), 7.35 (m, 2H, Ar), 14.46 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 20.0 (CH_3), 36.9 (C-5), 46.7 (C-4, C-6), 109.2 (NHC=C), 116.5, 116.9, 121.5, 127.2, 127.4, 127.7, 129.2, 129.5, 144.2, 144.5 (Ar), 174.4 (NHC=C). Anal. calc. for ($C_{20}H_{20}N_2O_2$): C, 74.98; H, 6.29; N, 8.74. Found: C, 74.78; H, 6.02; N, 8.53.

2-(1-((2-(Methylamino)phenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (18b). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, reaction of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (100 mg, 0.43 mmol) and N^1-methylbenzene-1,2-diamine (**17b**) (49 µL, 0.43 mmol) in toluene afforded **18b (**110 mg, 76%) as a yellow solid. Mp 144-146 ºC. MS (ES, positive mode): m/z 335 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 2.34 (s, 3H, CH_3), 2.58 (m, 2H, H-4, H-6), 2.64 (m, 2H, H-4, H-6), 2.70 (d, 3H, *J* = 4.8 Hz, NHCH_3) 3.39 (m, 1H, H-5), 5.40 (q, 1H, *J* = 4.5 Hz, NHCH_3),

6.67 (m, 2H, Ar), 7.01 (dd, 1H, *J* = 7.6, 1.2 Hz, Ar), 7.23 (m, 2H, Ar), 7.35 (d, 4H, *J* = 4.4 Hz, Ar), 14.46 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 19.3 (CH_3), 29.7 ($NHCH_3$), 36.2 (C-5), 46.0 (C-4, C-6), 108.6 (NHC=C), 110.7, 115.6, 121.3, 126.5, 126.7, 126.8, 128.5, 129.3, 143.5, 144.7 (Ar), 174.1 (NHC=C). Anal. calc. for ($C_{21}H_{22}N_2O_2$): C, 75.42; H, 6.63; N, 8.38. Found: C, 75.44; H, 6.40; N, 8.19.

2-(1-((2-Ethoxyphenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (20a). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (62 mg, 0.27 mmol) and *o*-phenetidine (**19a**) (53 µL, 0.41 mmol) in toluene afforded **20a** (69 mg, 76%) of as a white solid. Mp 125-127 ºC. MS (ES, positive mode): m/z 350 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 1.30 (t, 3H, *J* = 6.9 Hz, CH_3CH_2O), 2.43 (s, 3H, CH_3), 2.61 (m, 2H, H-4, H-6), 2.82 (m, 2H, H-4, H-6), 3.35 (m, 1H, H-5), 4.11 (q, 2H, *J* = 7.0 Hz, CH_3CH_2O), 7.02 (td, 1H, *J* = 7.6, 1.3 Hz, Ar), 7.18 (m, 1H, Ar), 7.24 (m, 1H, Ar), 7.33 (m, 6H, Ar), 14.81 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 14.5 (CH_3CH_2O), 19.7 (CH_3), 36.1 (C-5), 45.9 (C-6, C-4), 64.0 (CH_3CH_2O), 108.5 (NHC=C), 113.37, 120.6, 124.8, 126.5, 126.7, 126.9, 128.5, 129.1, 143.5, 152.2 (Ar), 172.3 (NHC=C). Anal. calc. for ($C_{22}H_{23}NO_3$): C, 75.62; H, 6.63; N, 4.01. Found: C, 75.47; H, 6.61; N, 3.89.

5-Phenyl-2-(1-((2-propoxyphenyl)amino)ethylidene)cyclohexane-1,3-dione (20b). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, reaction of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (62 mg, 0.27 mmol) and 2-propoxyaniline (**19b**) (61 mg, 0.41 mmol) in toluene yielded **20b** (64 mg, 65%) as a white solid. Mp 120-122 ºC. MS (ES, positive mode): m/z 364 $(M+H)^+$. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.01 (t, 3H, *J* = 7.4 Hz, $CH_3CH_2CH_2O$), 1.81 (m, 2H, *J* = 7.4 Hz, $CH_3CH_2CH_2O$), 2.52 (s, 3H, CH_3), 2.73 (m, 2H, H-4, H-6), 2.83 (m, 2H, H-4, H-6), 3.39 (tt, 1H, *J* = 11.8, 4.7 Hz, H-5), 3.97 (t, 2H, *J* = 6.6 Hz, $CH_3CH_2CH_2O$), 6.98 (m, 2H, Ar), 7.12 (ddd, 1H, *J* = 8.4, 6.8, 1.4 Hz, Ar), 7.25 (m, 1H, Ar), 7.27 (m, 2H, Ar), 7.30 (m, 1H, Ar), 7.35 (m, 2H, Ar), 14.67 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 10.6 ($CH_3CH_2CH_2O$), 20.6 (CH_3), 22.6 ($CH_3CH_2CH_2O$), 37.1 (C-5), 45.9, 47.3 (C-6, C-4), 70.3 ($CH_3CH_2CH_2O$), 109.2 (NHC=C), 112.8, 120.6, 125.7, 126.8, 127.1, 128.9, 129.2, 143.3, 153.4, 165.2 (Ar), 174.0 (NHC=C) 196.5, 199.1 (CO). Anal. calc. for ($C_{23}H_{25}NO_3$): C, 76.01; H, 6.93; N, 3.85. Found: C, 76.28; H, 6.78; N, 4.04.

2-(1-((2-Isopropoxyphenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (20c). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of 2-acetyl-5-

phenylcyclohexane-1,3-dione (**12**)[10] (51 mg, 0.22 mmol) and 2-isopropoxyaniline (**19c**) (50 mg, 0.33 mmol) in toluene afforded **20c** (52 mg, 66%) as a white solid. Mp 89-91 ºC. MS (ES, positive mode): m/z 364 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 1.26 (d, 6H, *J* = 6.0 Hz, OCH($C\underline{H}_3$)$_2$), 2.44 (s, 3H, CH_3), 2.61 (m, 2H, H-4, H-6), 2.82 (m, 2H, H-4, H-6), 3.34 (m, 1H, H-5), 4.66 (hept, 1H, *J* = 6.0 Hz, OC$\underline{H}$(CH_3)$_2$), 7.01 (td, 1H, *J* = 7.7, 1.1 Hz, Ar), 7.21 (m, 1H, Ar), 7.23 (m, 1H, Ar), 7.31 (m, 1H, Ar), 7.33 (m, 5H, Ar), 14.82 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 20.0 (CH_3), 21.8 (OCH($\underline{C}H_3$)$_2$), 36.1 (C-5), 45.9 (C-6, C-4), 71.0 (O$\underline{C}$H(CH_3)$_2$), 108.5 (NHC=$\underline{C}$), 115.1, 120.6, 125.8, 126.5, 126.8, 126.8, 128.5, 128.9, 143.6, 151.3, (Ar), 172.0 (NH$\underline{C}$=C). Anal. calc. for ($C_{23}H_{25}NO_3$): C, 76.01; H, 6.93; N, 3.85. Found: C, 76.30; H, 7.02; N, 4.00.

2-(1-((2-(Cyclopropylmethoxy)phenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (20d). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (51 mg, 0.22 mmol) and 2-(cyclopropylmethoxy)aniline (**19d**)[30] (54 mg, 0.33 mmol) yielded **20d** (45 mg, 57%) as a rosaceous solid. Mp 76-78 ºC. MS (ES, positive mode): m/z 376 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 0.32 (m, 2H, H-2', H-3'), 0.53 (m, 2H, H-2', H-3'), 1.20 (m, 1H, H-1'), 2.44 (s, 3H, CH_3), 2.65 (m, 2H, H-4, H-6), 2.82 (m, 2H, H-4, H-6), 3.38 (m, 1H, H-5), 3.94 (d, 2H, *J* = 6.8 Hz, OCH_2), 7.03 (t, 1H, *J* = 7.7 Hz, Ar), 7.19 (m, 1H, Ar), 7.24 (m, 1H, Ar), 7.30 (m, 1H, Ar), 7.33 (m, 3H, Ar), 7.35 (m, 2H, Ar), 14.80 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 3.0 (C-2', C-3'), 9.9 (C-1'), 19.9 (CH_3), 36.1 (C-5), 45.9 (C-6, C-4), 72.8 (OCH_2), 108.5 (NHC=$\underline{C}$), 113.8, 120.7, 125.0, 126.5, 126.8, 126.8, 128.5, 129.1, 143.5, 152.4 (Ar), 172.4 (NH$\underline{C}$=C). Anal. calc. for ($C_{24}H_{25}NO_3$): C, 76.77; H, 6.71; N, 3.73. Found: C, 76.48; H, 6.50; N, 3.72.

2-(1-((2-(2-Hydroxyethoxy)phenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (22a). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (143 mg, 0.62 mmol) reacted with 2-(2-((*tert*-butyldimethylsilyl)oxy)ethoxy)aniline (**21a**)[14] (200 mg, 0.75 mmol) in toluene (6 mL). Volatiles were removed and the residue was dissolved in anhydrous THF (1.5 mL) and 1M TBAF in THF (410 µL, 0.41 mmol) was added. The reaction mixture was stirred at room temperature for 1h, quenched with water and extracted with dichloromethane (2x 15 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The crude was purified by flash chromatography (hexane/ethyl acetate) to yield **22a** (109 mg, 48% for the two steps) as a white solid. Mp 156-158 ºC. MS (ES, positive mode): m/z 366 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.44 (s, 3H, CH_3), 2.61 (m, 2H, H-6, H-4), 2.82 (m, 2H, H-6, H-4), 3.34

(m, 1H, H-5), 3.70 (m, 2H, $CH_2C\underline{H}_2OH$), 4.10 (t, 2H, J = 5.0 Hz, $C\underline{H}_2CH_2OH$), 4.81 (t, 1H, J = 5.3 Hz, OH), 7.03 (td, 1H, J = 7.4, 1.3 Hz, Ar), 7.23 (m, 2H, Ar), 7.30 (dd, 1H, J = 7.8, 1.7 Hz, Ar), 7.33 (m, 2H, Ar), 7.34 (m, 2H, Ar), 7.35 (m, 1H, Ar), 14.80 (br s, 1 H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 19.9 (CH_3), 36.1 (C-5), 45.6 (C-4, C-6), 57.9 ($CH_2\underline{C}H_2OH$), 65.2 ($\underline{C}H_2CH_2OH$), 108.9 (NHC=$\underline{C}$), 113.5, 120.7, 124.5, 124.9, 126.5, 126.8, 128.5, 129.1, 143.6, 152.5 (Ar), 172.5 (NH$\underline{C}$=C). Anal. calc. for ($C_{22}H_{23}NO_4$): C, 72.31; H, 6.34; N, 3.83. Found: C, 72.40; H, 6.53; N, 4.01.

2-(1-((2-(3-Hydroxypropoxy)phenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (22b). As described for compound **22a**, reaction of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (40 mg, 0.17 mmol) and 2-(3-((*tert*-butyldimethylsilyl)oxy)-propoxy)aniline (**21b**)[14, 15] (73 mg, 0.26 mmol) in toluene afforded a condensation product that was dissolved in anhydrous THF (1.3 mL) and treated with 1M TBAF (290 µL, 0.29 mmol). After stirring for 1h at room temperature, the mixture was quenched with water and extracted with dichloromethane (2x 15 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The crude was purified by flash chromatography (hexane/ethyl acetate) to yield **22b (**58 mg, 90%) as a white solid. Mp 83-85 ºC. MS (ES, positive mode): m/z 380 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 1.85 (m, 2H, J = 6.2 Hz, $CH_2C\underline{H}_2CH_2OH$), 2.44 (s, 3H, CH_3), 2.62 (m, 2H, H-6, H-4), 2.83 (m, 2H, H-6, H-4), 3.37 (m, 1H, H-5), 3.56 (m, 2H, $CH_2CH_2C\underline{H}_2OH$), 4.12 (t, 2H, J = 6.3 Hz, $C\underline{H}_2CH_2CH_2OH$), 4.62 (br s, 1H, OH), 7.02 (dt, 1H, J = 7.6, 1.2 Hz, Ar), 7.20 (dd, 1H, J = 8.4, 1.2 Hz, Ar), 7.23 (m, 1H, Ar), 7.33 (m, 6H, Ar), 14.79 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 20.3 (CH_3), 32.7 ($CH_2\underline{C}H_2CH_2OH$), 36.6 (C-5), 45.9 (C-6, C-4), 59.4 ($CH_2CH_2\underline{C}H_2OH$), 70.3 ($\underline{C}H_2CH_2CH_2OH$), 108.5 (NHC=$\underline{C}$), 112.2, 114.3, 116.6, 127.2, 128.9, 138.2, 144.0, 147.0, 146.1, 152.8, (Ar), 172.7 (NH$\underline{C}$=C). Anal. calc. for ($C_{23}H_{25}NO_4$): C, 72.80; H, 6.64; N, 3.69. Found: C, 72.53; H, 6.92; N, 3.99.

2-(1-((2-(2-(2-Hydroxyethoxy)ethoxy)phenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (22c). As described for compound **22a**, reaction of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (40 mg, 0.17 mmol) and 2-(2-(2-((*tert*-butyldimethylsilyl)oxy)ethoxy)ethoxy)aniline (**21c**)[14, 15] (81 mg, 0.26 mmol) in toluene afforded a condensation product that was dissolved in anhydrous THF (1.3 mL) and treated with 1M TBAF (290 µL, 0.29 mmol). After stirring for 1h at room temperature, the mixture was quenched with water and extracted with dichloromethane (2x 15 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The crude was purified by flash chromatography (hexane/ethyl acetate) to yield **22c** (40 mg, 57%) as a white solid. Mp 105-107 ºC. MS (ES, positive mode): m/z 410 $(M+H)^+$. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.43 (s, 3H, CH_3), 2.61 (m, 2H, H-6, H-4), 2.82 (m, 2H, H-6, H-4), 3.30 (m, 1H, H-5), 3.47-

3.72 (m, 6H, $CH_2C\underline{H}_2OC\underline{H}_2C\underline{H}_2OH$), 4.18 (m, 2H, $C\underline{H}_2CH_2OCH_2CH_2OH$), 4.59 (t, 1H, *J* = 5.3 Hz, OH), 7.04 (dt, 1H, *J* = 7.6, 1.2 Hz, Ar), 7.23 (m, 2H, Ar), 7.33 (m, 6H, Ar), 14.79 (br s, 1 H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 19.9 (CH_3), 36.2 (C-5), 46.0 (C-4, C-6), 60.2 ($CH_2CH_2OCH_2\underline{C}H_2OH$), 68.2, 68.7 ($CH_2\underline{C}H_2O\underline{C}H_2CH_2OH$), 72.6 ($\underline{C}H_2CH_2OCH_2CH_2OH$), 108.5 (NHC=$\underline{C}$), 113.4, 120.8, 124.5, 124.9, 126.6, 126.8, 128.6, 129.2, 143.6, 152.4 (Ar), 172.7 (NH$\underline{C}$=C). Anal. calc. for ($C_{24}H_{27}NO_5$): C, 70.40; H, 6.65; N, 3.42. Found: C, 70.26; H, 6.45; N, 3.45.

2-(1-((2-(2-(2-Aminoethoxy)ethoxy)phenyl)amino)ethylidene)-5-phenylcyclo-hexane-1,3-dione (22d). As described for compound **22a**, reaction of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (220 mg, 0.78 mmol) and *tert*-butyl(2-(2-(2-aminophenoxy)ethoxy)ethyl)carbamate (**21d**)[16] (466 mg, 1.17 mmol) in toluene afforded 360 mg of the condensation product. MS (ES, positive mode): m/z 509 $(M+H)^+$. A solution of this compound (300 mg, 0.60 mmol) in $CHCl_3$ (6 mL) was treated with TFA (450 µL, 6.00 mmol) at room temperature overnight. Volatiles were removed in vacuo yielding **22d** (288 mg, 84 % yield, two steps) as a white solid (trifluoroacetate salt). Mp 148-150 ºC. MS (ES, positive mode): m/z 409 $(M+H)^+$. ^{1}H NMR (methanol-d_4, 500 MHz) δ: 2.56 (s, 3H, CH_3), 2.78 (m, 2H, H-4, H-6), 2.88 (m, 2H, H-4, H-6), 3.15 (m, 2H, $NHC\underline{H}_2CH_2$), 3.39 (m, 1H, H-5), 3.74 (m, 2H, $NHCH_2C\underline{H}_2$), 3.89 (m, 2H, $OC\underline{H}_2CH_2OPh$), 4.27 (m, 2H, $CH_2C\underline{H}_2OPh$), 7.09 (dt, 1H, *J* = 7.7, 1.2 Hz, Ar), 7.18 (dd, 1H, *J* = 8.4, 1.2 Hz, Ar), 7.26 (m, 1H, Ar), 7.30 (dd, 1H, *J* = 7.9, 1.6 Hz, Ar), 7.34 (m, 4H, Ar), 7.40 (ddd, 1H, *J* = 8.3 7.6, 1.6 Hz, Ar). Anal. calc. for ($C_{26}H_{29}F_3N_2O_6$): C, 59.77; H, 5.59; N, 5.39. Found: C, 59.50; H, 5.30; N, 5.42.

2-(1-((2-(2-Morpholinoethoxy)phenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (24). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (58 mg, 0.25 mmol) and 2-[2-(morpholin-4-yl)ethoxy]aniline (**23**)[19] (37 mg, 0.17 mmol) reacted in toluene at 110 ºC overnight. After workup, the residue was purified by CCTLC (dichloromethane/methanol 10/1) to yield **24** (24 mg, 32%) as a brown oil. MS (ES, positive mode): m/z 435 $(M+H)^+$ ^{1}H NMR (DMSO-d_6, 400 MHz) δ: δ 2.39 – 2.45 (m, 7H, CH3, H-3', H-5'), 2.56 – 2.63 (m, 2H, H-4, H-6), 2.66 (t, J = 5.5 Hz, 2H, NCH2), 2.72 – 2.90 (m, 2H, H-4, H-6), 3.30 (m, 1H, H-5), 3.50 – 3.54 (m, 4H, H-2', H-6'), 4.16 (t, J = 5.4 Hz, 2H, OCH_2), 7.03 (td, J = 7.6, 1.2 Hz, 1H, Ar), 7.19 – 7.27 (m, 2H, Ar), 7.27 – 7.40 (m, 6H, Ar), 14.73 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 19.8 (CH_3), 36.2 (C-5), 43.1 (C-4, C-6), 53.6 (C3',C5'), 56.8 (NCH_2), 66.2 (C2',C6'), 66.6 (OCH_2), 108.4 (NHC=$\underline{C}$), 113.4, 120.8, 124.9, 126.5, 126.8, 126.9, 128.5, 129.2, 143.5, 152.4 (Ar), 172.5 (NH$\underline{C}$=C). Anal. calc. for ($C_{26}H_{30}N_2O_4 \cdot 0.5H_2O$): C, 70.41; H, 7.05; N, 6.32. Found: C, 70.70; H, 7.09; N, 6.70.

2-((1-(3-((*Tert*-butyldimethylsilyl)oxy)propyl)piperidin-4-yl)oxy)aniline (25). To a mixture containing 4-(2-nitrophenoxy)piperidine (372 mg, 1.11 mmol) and Na_2CO_3 (118 mg, 1.11 mmol) in acetone (4.4 mL), 3-bromopropanol (194 µL, 2.22 mmol) was added dropwise. The reaction was stirred at 50 ºC for 24 h under argon atmosphere. After cooling, a saturated aqueous NH_4Cl solution (20 mL) was added and extracted with dichloromethane (2x20 mL). The combined organic fractions were dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by CCTLC (dichloromethane:ammonia solution 7 N in methanol, 10:0.2) to yield 3-(4-(2-nitrophenoxy)piperidin-1-yl)propan-1-ol (230 mg, 74%) as a yellow oil. MS (ES, positive mode): m/z 281 $(M+H)^+$. To a solution of this alcohol (210 mg, 0.75 mmol) in DMF anhydrous, *tert*-butyldimethylsilyl chloride (159 mg, 1.12 mmol), dimethylaminopyridine (92 mg, 0.075 mmol) and imidazole (102 mg, 1.50 mmol) were added. The reaction mixture was stirred at rt for 16 h under argon atmosphere, concentrated in vacuo, washed with NH_4Cl and extracted with dichloromethane (2x20 mL). The combined organic fractions were dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (dichloromethane/methanol, 10/1) to yield the protected alcohol (293 mg, 99%) of [MS (positive mode): m/z 395 $(M+H)^+$] that was used for the next step. A solution of the protected alcohol (293 mg, 0.74 mmol) in ethanol (12 mL) in the presence of 10 % Pd/C (catalytic amount) was hydrogenated (30 psi) for 5 h at 30 ºC. Then, the reaction mixture was filtered and volatiles were removed to yield **25** (267 mg, 99%) of as rosaceous oil that was pure enough for the next step. MS (ES, positive mode): m/z 365 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.87 (s, 9H, $(CH_3)_3$), 1.67 (m, 4H, $CH_2C\underline{H}_2CH_2O$, H-3', H-5'), 1.92 (m, 2H, H-3', H-5'), 2.35 (m, 2H, H-2', H-6'), 2.45 (m, 2H, $C\underline{H}_2CH_2CH_2O$), 2.70 (m, 2H, H-2', H-6'), 3.62 (t, 2H, J = 6.2 Hz, $CH_2CH_2C\underline{H}_2O$), 4.26 (m, 1H, H-4'), 4.65 (br s, 2H, NH_2), 6.47 (m, 1H, Ar), 7.64 (m, 2H, Ar), 6.81 (m, 1H, Ar).

2-(1-((2-((1-(3-Hydroxypropyl)piperidin-4-yl)oxy)phenyl)amino)ethylidene)-5-phenylcyclohexane-1,3-dione (26). Following the general procedure for the reaction of 2-acylcyclohexane-1,3-diones with anilines, a solution of 2-acetyl-5-phenylcyclohexane-1,3-dione (**12**)[10] (155 mg, 0.68 mmol) reacted with **25** (164 mg, 0.45 mmol) in toluene. After cooling, the solvent was evaporated to dryness and the residue was dissolved in dichloromethane (1.4 mL) and TFA (1.4 mL). The reaction mixture was stirred at rt for 1h and volatiles were removed. The residue was purified by CCTLC (dichloromethane/ methanol 10/1) to yield **26** (102 mg, 49%) as a white solid. Mp 104-106º C. MS (ES, positive mode): m/z 463 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.54 (m, 2H, $CH_2C\underline{H}_2CH_2O$), 1.60 (m, 2H, H-3', H-5'), 1.85 (m, 2H, H-3', H-5'), 2.24 (m, 2H, H-2', H-6'), 2.32 (t, 2H, J = 7.4 Hz, $C\underline{H}_2CH_2CH_2O$), 2.42 (s, 3H, CH_3), 2.53 (m, 2H, H-2', H-6'), 2.61 (m, 2H, H-4, H-6),

2.80 (m, 2H, H-4, H-6), 3.28 (m, 1H, H-5), 3.40 (t, 2H, J = 6.3 Hz, $CH_2CH_2CH_2O$), 4.51 (m, 2H, H-4', OH), 7.02 (m, 1H, Ar), 7.23 (m, 2H, Ar), 7.31 (m, 6H, Ar), 14.80 (br s, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 20.0 (CH_3), 29.7 (CH_2), 30.2 (C-3', C-5'), 36.2 (C-5), 40.1 (C-2', C-6'), 49.5 (C-4, C-6), 55.1 (CH_2), 59.5 (CH_2), 72.9 (C-4'), 108.5 (NHC=C), 115.3, 120.8, 126.1, 126.5, 126.8, 127.0, 128.5, 128.9, 143.5, 150.1 (Ar), 172.2 (NHC=C). Anal. calc. for ($C_{28}H_{34}N_2O_4 \cdot 0.5H_2O$): C, 71.31; H, 7.48; N, 5.94. Found: C, 71.36; H, 7.60; N, 6.10.

Biological methods

Cell proliferation.

Endothelial cells. Bovine aortic endothelial cells (BAEC) and human microvascular endothelial cells (HMEC-1) were seeded in 48-well plates at 10,000 cells/well or 20,000/well), respectively. After 24h, 5-fold dilutions of the compounds were added. The cells were allowed to proliferate 3 or 4 days for BAEC and HMEC-1, respectively, in the presence of the compounds, trypsinized, and counted by means of a Coulter counter (Analis, Belgium).

Tumor cells. Human cervical carcinoma (HeLa) cells were seeded in 96-well plates at 15,000 cells/well in the presence of different concentrations of the compounds. After 4 days of incubation, the cells were trypsinized and counted in a Coulter counter. Suspension cells (Mouse leukemia L1210 and human lymphoid CEM cells) were seeded in 96-well plates at 60,000 cells/well in the presence of different concentrations of the compounds. L1210 and CEM cells were allowed to proliferate for 48 h or 96 h, respectively and then counted in a Coulter counter. The 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce cell proliferation by 50%.

Cell cycle analysis. HMEC-1 cells were seeded in 6-well plates at 125,000 cells/well in DMEM with 10 % FBS. After 24 h, the cells were exposed to different concentrations of the compounds. After 16h, the DNA of the cells was stained with propidium iodide using the CycleTEST PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA). The DNA content of the stained cells was assessed by flow cytometry on a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences) within 3h after staining. Cell debris and clumps were excluded from the analysis by appropriate dot plot gating. Percentages of sub-G1, G1, S, and G_2/M cells were estimated using appropriate region markers.

Tube destruction. Wells of a 96-well plate were coated with 70 µl matrigel (10 mg/ml, BD Biosciences, Heidelberg, Germany) at 4°C. After gelatinization at 37°C during 30 min, HMEC-1 cells were seeded at 60,000 cells/well on top of the matrigel in 200 µl DMEM containing 10% FCS. After 3h of incubation at

37°C, when the endothelial cells had reorganized to form tube-like structures, the compounds were added. Two hours later,the cultures were photographed at 4 x magnification. Tube formation was quantified by giving a score from 0 (no tubes) to 3 (complete vascular network, as seen in control cultures without compound).

Tubulin binding. Human breast carcinoma MDA-MB-231 cells were seeded in 6-well plates at 500,000 cells/well. After 48h, compounds were added to the cells for 16h before adding EBI (N,N'-ethylene-bis(iodoacetamide) at 100 μM. After 1.5h, the cells were harvested and cell extracts were prepared for western blot analysis. Twenty μg of proteins were subjected to gel electrophoresis using 0.1% SDS (85% purity) and 10 % polyacrylamide gels. After electrophoresis, proteins were transferred to pretreated Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were incubated for 1 h at room temperature in blocking buffer (2.5 % non-fat dry milk in PBS containing 0.1% Tween) and subsequently for 16 h at 4°C in blocking buffer with primary antibodies raised against β-tubulin. After washing, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody in blocking buffer for 25' at room temperature. Next, the membranes were washed extensively. Immunoreactive proteins were detected by chemiluminescence (ECLplus, Bio-Rad).

Determination of binding constants.

Proteins and ligands Calf brain tubulin was purified as described.[31] (R)-(+)-ethyl 5-amino 2-methyk-1,2-dihydro-3-phenylpyrido[3,4-b]pyrazin-7-yl carbamate (R-PT)[20] was a kind gift of Prof. G.A., Rener Organic Chemistry Research Department, Southern Research Institute, Birmingham, Alabama. The compounds were diluted in 99.8% D6-DMSO (Merck, Darmstadt, Germany) to a final concentration of 10 mM and stored at -80 ºC.

Determination of binding constants. R-PT (Ka 5.1×10^6 M^{-1})[10] was used as a reference ligand as described in reference.[32] For that purpose, the fluorescence emission of a previous mixed sample of 0.2μM of R-PT and 0.2 μM of tubulin was evaluated in presence of increasing concentrations of studied ligand in a black 96-well plate (0; 0.05; 0.2; 0.5; 2; 5; 10; 30; 50; 70 μM). The samples were incubated 30 minutes at 25ºC in a *Varioskan* plate reader (Thermo Scientific Waltham, Massachusetts, USA) before the fluorescence emission intensity at 456 nm (excitation 374 nm) was measured. The data were analyzed and the binding constants determined using Equigra V5.0.[31]

Solubility determination.

Excess amount of the tested compound was added to 400 µL of PBS buffer with 1% DMSO, and the resulting suspension was shaken at room temperature for 2 h on a rotary shaker. The samples were centrifuged at 135 rpm in a Hettich microcentrifuge for 15 min at room temperature. Finally, 160 µL of the clear supernatant were transferred in a quartz microplate and were diluted by adjunction of 40 µL of CH_3CN:DMSO 8:2 solution. The solubility was determined using UV detection and comparison with calibration standards previously prepared. Standards are made in an PBS:CH_3CN (8:2) solution to ensure overall compound solubility. Additionally, the level of DMSO in all calibrators is maintained at 5% (v/v) ensuring that the final solvent content of all standards and samples remains consistent. For the preparation of standards, the PBS:CH_3CN solution and DMSO were added to the plate and mixed thoroughly before the addition of 10 mM DMSO stock compound. Concentration of the 5 standard calibrators were 10 µM, 100 µM, 250 µM, 350 µM and 500 µM. A standard solution with a known concentration was used to validate the calibration curve.

Computational studies

Docking of 20a. Compound **20a** was used as ligand for the automated docking experiments. DAMA-colchicine-tubulin complex (Protein Data Bank code: 1SA0)[23] and TN-16-tubulin complex (PDB ID: 3HKD)[9] were retrieved from the Protein Data Bank.[33] AMBER-compatible RESP point charges were used for **20a**. The Lamarkian genetic algorithm implemented in AutoDock 4.0.5[24] was used to generate the docked conformations within the putative binding cavity by randomly changing the overall orientation of the molecule as well as the torsion angles of all rotable bonds. Default settings were used except for the number of runs, population size, and maximum number of energy evaluations, which were fixed at 250, 100 and 250.000, respectively. Rapid intra- and intermolecular energy evaluations of each configuration was achieved by having the receptor´s atomic affinity potentials for aliphatic and aromatic carbon, oxygen, nitrogen and hydrogen atoms precalculated in a three-dimensional grid with a spacing of 0.375 Å. A distance-dependent dielectric function was used in the computation of electrostatic interactions.

Conflict of interest.

The authors declare no financial or commercial conflict of interest.

Abbreviations

VDA: vascular-disrupting agent; TN-16: 3-[1-(Phenylamino)ethylidene]-5-(phenylmethyl)-2,4-pyrrolidinedione ;EBI : *N,N'*-ethylene-bis(iodoacetamide);R-PT: (R)-(+)-ethyl 5-amino 2-methyl-1,2-dihydro-3-phenylpyrido[3,4-*b*]pyrazin-7-yl carbamate.

Acknowledgments.

M.-D. C. thanks the Fondo Social Europeo (FSE) and the JAE Predoc Programme for a predoctoral fellowship. This project has been supported by the Spanish Ministerio de Economía y Competitividad (SAF2012-39760-C02-01 to E.-M. P, M.-J. P.-P. and M.-J. C and BIO2013-42984-R to J. F. D.), and Comunidad de Madrid (BIPEDD2; ref. P2010/BMD-2457 to M.-J. C and J. F. D.). J. F. D. acknowledges networking contribution by the COST Action CM1407 "Challenging organic synthesis inspired by nature-from natural products chemistry to drug discovery" and the COST action CM1470. We also wish to thank Eef Meyen and Lizette van Berckelaer for excellent technical assistance.

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Tatgerting the colchicine site in tubulin through cyclohexanedione derivatives

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