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Novel amino analogs of the trimethoxyphenyl ring in potent colchicine site ligands improve solubility by the masked polar group incorporation (MPGI) strategy

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ABSTRACT

The low aqueous solubility of colchicine site antimitotic agents, of which the trimethoxyphenyl (A ring) is a heavy contributor, is a serious drawback in their clinical development. We have designed new A ring analogs with chameleonic masked polar amino groups able to increase aqueous solubility and also behave as non-polar through intramolecular hydrogen bonds when bound to tubulin. We have incorporated these new A rings in several scaffolds (sulfonamides, combretastatins, phenstatins, isocombretastatins), synthesized, and assayed 43 representatives. The amino analogs show improved aqueous solubility and some of them (**8**, **60Z**, and **67**) nanomolar anti-proliferative potencies against human cancer cell lines, with the most favorable substituent being a 3-methylamino group. The antiproliferative effect relates to tubulin inhibition as shown by in vitro tubulin polymerization inhibition, immunofluorescence microscopy, and cell cycle and apoptosis analysis by flow cytometry. The compounds arrest the cell cycle of treated cells in G_2/M and later develop an apoptotic response. Docking studies suggested binding at the colchicine site of tubulin with good agreement with the DFT models of the new structural variations made. The 3-methylamino-4,5-dimethoxyphenyl moiety is an example of the masked polar group incorporation (MPGI) strategy for soluble ligands binding to hydrophobic sites and a good trimethoxyphenyl ring replacement for the development of new colchicine site ligands.

1. Introduction

The microtubules of eukaryotic cells, the tubulin dimers that form them, and their complex polymerization – depolymerization equilibria are one of the most successful targets of chemotherapeutic antitumor drugs, such as the taxanes or the Vinca minor alkaloids.[1] However, tumors eventually develop resistance toward these large and hydrophobic natural products, often by developing multidrug resistance (MDR) phenotypes based on efflux pumps such as the P-glycoprotein.[2] Therefore, new drugs targeting tubulin are actively being sought, especially those with more drug-like properties. In the search for new anti-tubulin drugs, alternatives to taxane or vinca binding sites are preferred. They foresight a bigger susceptibility to drug-like compounds conferred by smaller sizes, which might as well help in the avoidance of the MDR efflux pumps and facilitate synthetic access.[3] The colchicine site of tubulin is located at the interface between the α and β tubulin subunits of the tubulin dimer.[4] Many compounds have been shown to bind to the colchicine site, most of them composed of two aryl systems connected by different linkers (alkenes as in combretastatins or isocombretastatins, ketones in phenstatins or nocodazole, sulfonamides as in ABT-751, amines as in verubulin, and others).[5] Structural studies have shown that the colchicine site is composed of three subpockets (B-A-C), and most ligands bind to two of them with their aromatic rings.[3,6–8] Due to the hydrophobic nature of the colchicine site, solubility is

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a major concern for colchicine site ligands, and solubilizing moieties have been introduced on the active skeletons to increase the polarity and hydrophilicity, either directly or via prodrugs, as in the combretastatin A-4 phosphate prodrug (CA4P, fosbretabulin). Most of these modifications have been carried out on the B-rings, as they are more tolerant to structural variations.[5] However, the hydroxyl group of combretastatin A-4 or its phosphate prodrug render the drugs susceptible to phase II metabolic transformation by UDP-glycosyltransferases, thus leading to resistance of cancer cells.[9] As an alternative solution to hydroxyl groups, amino groups have been introduced on the B rings and shown to afford potent and more soluble analogs, such as in amines crolibulin, verubulin, or the aminocombretastatin prodrug ombrabulin, and we have recently applied it in sulfonamides.[5,10].

Structural modifications aimed at improving the aqueous solubility of colchicine site inhibitors directed towards the central A subpocket of the colchicine site are much scarce,[11–14] where the trimethoxyphenyl ring of so-called classical colchicine site ligands is highly prevalent.[15] With this aim, we have successfully explored the possibility of substituting the A phenyl ring by substituted pyridines.[16–18] For combretastatins, replacement of one of the lateral methoxy groups of the trimethoxyphenyl ring by an amino group has been explored for B rings of trimethoxyphenyl,[19,20] naphthalene,[14] and 3-amino-4-methoxyphenyls.[13] The approach has also been explored in non-isomerizable heterocyclic combretastatin analogs.[12] Here, we pursued the exploration of introducing amino groups on our previously described methoxybenzene sulfonamides with differently substituted A phenyl rings[21] and the effect of substitutions on the amine in the search for novel colchicine site ligands with improved pharmacokinetic properties. We have further extended these studies to other bridges such as olefins and ketones with concomitant variations on the B ring. The overall design process is summarized in Scheme 1.

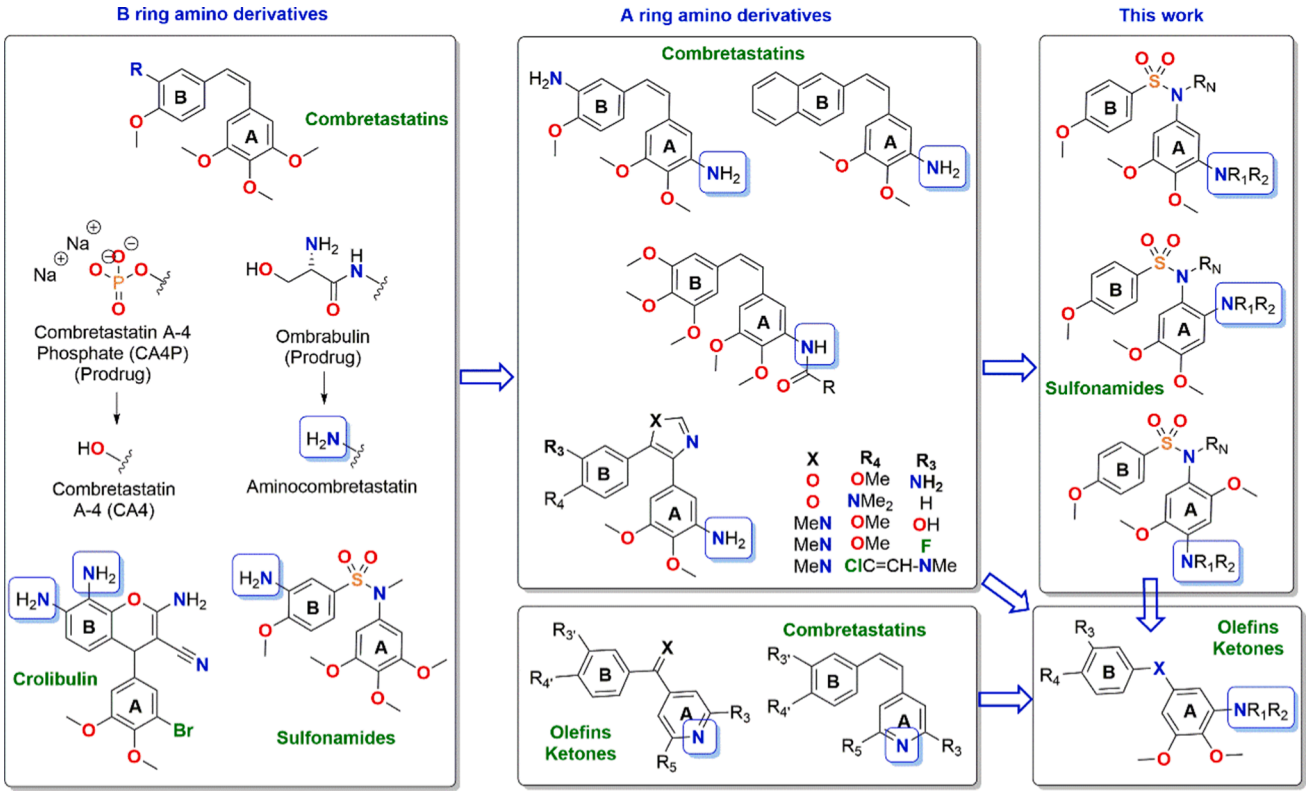
2. Results and discussion

2.1. Chemistry

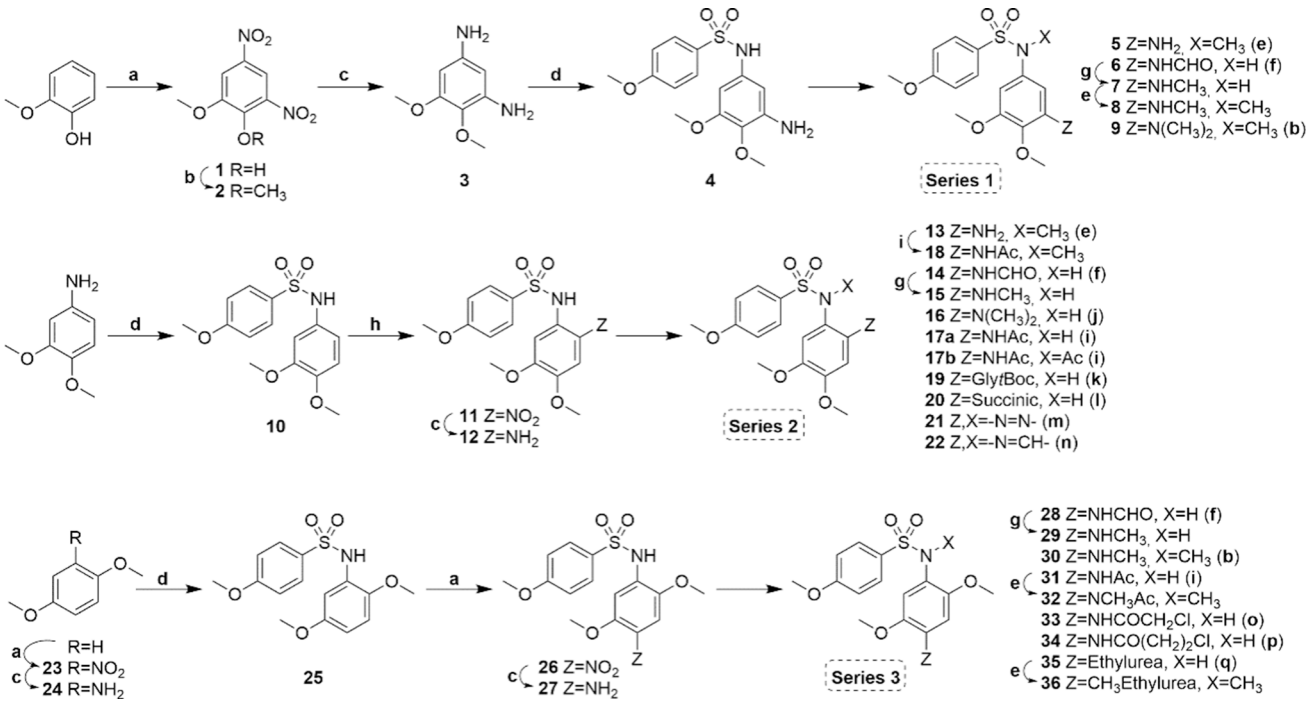
2.1.1. Chemical synthesis

New derivatives were synthetized following the synthetic approaches outlined in Schemes 2-3. Compounds have been classified (Table 1) into four different series (1–4) according to the bridge between both aromatic rings (sulfonamides for series 1–3 and olefins or ketones for series 4) and the substitution pattern of the aniline (A ring) in the sulfonamides (3-amino-4,5-dimethoxy for series 1, 2-amino-4,5-dimethoxy for series 2, and 4-amino-2,5-dimethoxy for series 3). Different synthetic methodologies were applied depending on the bridge nature for the assemblage of the skeletons, either sulfonamide (series 1–3) or combretastatin, phenstatin and isocombretastatin analogs (series 4).

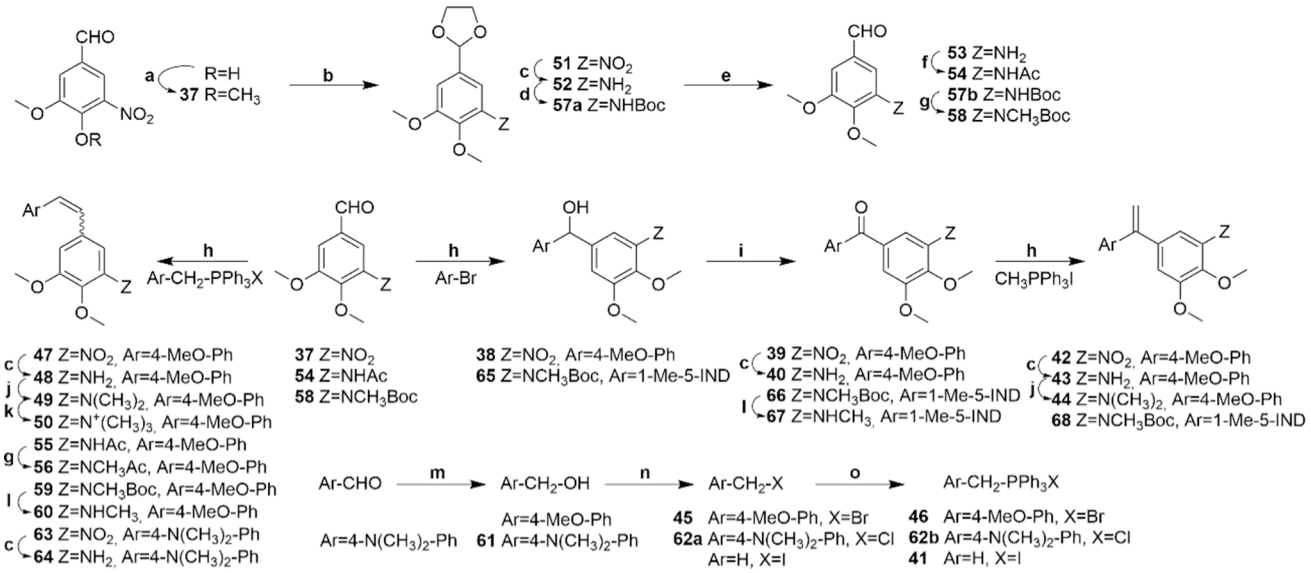
The diarylsulfonamides were built up in good yields by reaction between the corresponding anilines with 4-methoxybenzenesulfonyl chloride (**4**, **10**, and **25**). Anilines for series 1 (**3**) and 3 (**24**) were synthesized by nitration-reduction sequences. After sulfonamide assembly, the amine substituents were introduced on the A rings by selective *ortho* to sulfonamido nitration with *tert*-butyl nitrite (**11**), or *para* with nitric acid (**26**), and subsequent palladium-catalyzed hydrogenation (**12** and **27**, respectively). For series 1, phenylenediamine **3** already carries the desired amino group in place at the sulfonamide formation step. Diarylsulfonamides with primary amino groups (**4**, **12**, and **27**) were further derivatized by formylation (**6**, **14**, and **28**), dimethylation (**9**, **16**, and **30**), acetylation (**17**, **18**, and **31**), and acylation (**19**, **20**, and **33**–**35**) reactions. Selective aniline monomethylation leaving the sulfonamide nitrogen unsubstituted was successfully achieved by formylation (**6**, **14**, and **28**) followed by reduction with sodium borohydride and trichloroacetic acid (**7**, **15**, and **29**, respectively). Methylations with dimethyl sulfate in acetone and K_2CO_3 (**9** and **30**) affected both the sulfonamides and the anilines. Dimethylamine derivatives were achieved by reductive amination with excess paraformaldehyde in



Scheme 1. Design of new A-ring amino derivatives.



Scheme 2. Chemical synthesis of compounds in series 1–3. Reagents, conditions, and yields: (**a**) HNO_3, AcOH, 0 °C, 1–4 h, 90–94 %. (**b**) $(CH_3)_2SO_4$, K_2CO_3, acetone, reflux, 12–48 h, 15–98 %. (**c**) H_2, Pd (C), EtOAc, 48–72 h, 82–99 %. (**d**) 4-Methoxybenzenesulfonyl chloride, pyridine, CH_2Cl_2, 4–12 h, 73–99 %. (**e**) CH_3I, KOH, CH_3CN, 24 h, 52–98 %. (**f**) Formic acid, pyridine, CH_2Cl_2, 48–72 h, 67–89 %. (**g**) Trichloroacetic acid, $NaBH_4$, dry THF, 0 °C, 24–48 h, 34–93 %. (**h**) *tert*-Butyl nitrite, CH_3CN, 45 °C, 24 h, 95 %. (**i**) Acetic anhydride, pyridine, CH_2Cl_2, 30 min-24 h, 32–90 %. (**j**) Paraformaldehyde, $NaBH_3CN$, AcOH, MeOH, 96 h, 40 %. (**k**) Succinic anhydride, pyridine, CH_2Cl_2, 24 h, 59 % (**l**) (*tert*-butoxycarbonyl)glycine, EDCI, 4-DMAP, CH_2Cl_2, 24 h, 30 %. (**m**) *tert*-Butyl nitrite, CH_3CN, H_2O, AcOH, 0 °C, 24 h, 29 %. (**n**) Triethyl orthoformate, CH_3CN, reflux, 12 h, 97 %. (**o**) 2-Chloroacetyl chloride, CH_2Cl_2, 12 h, 87 %. (**p**) 3-Chloropropanoyl chloride, CH_2Cl_2, 12 h, 48 %. (**q**) Ethyl isocyanate, CH_2Cl_2, 6 h, 73 %.



Scheme 3. Chemical synthesis of compounds in series 4. Reagents, conditions, and yields: (**a**) $(CH_3)_2SO_4$, K_2CO_3, acetone, reflux, 48 h, 81 %. (**b**) $HO(CH_2)_2OH$, TsOH, toluene, Dean-Stark, 120 °C, 24 h, 96 %. (**c**) Zn powder, AcOH, CH_2Cl_2, 0 °C, 4–24 h, 69–98 %. (**d**) Boc_2O, THF, reflux, 48 h, 81 %. (**e**) HCl/H_2O, MeOH, 4 h, 97 %. (**f**) Ac_2O, pyridine, CH_2Cl_2, 2 h, 72 %. (**g**) CH_3I, NaH, THF, 15 h, 75–90 %. (**h**) *n*BuLi, dry THF, −78 °C to room temperature, 24 h, 47–97 %. (**i**) PDC, CH_2Cl_2, 0 °C, 4 h, 37–83 %. (**j**) CH_3I, acetone, reflux, 24 h, 88–97 %. (**k**) CH_3I, $AgNO_3$, acetone, 24 h, 49 %. (**l**) TMSCl, MeOH, 24 h, 56–88 %. (**m**) $NaBH_4$, MeOH, 2 h, 95 %. (**n**) X = Br: PBr_3, Et_2O, -40 °C, 4 h, 98 %; X = Cl: HCl(g), CH_2Cl_2. (**o**) PPh_3, toluene, 15–24 h, 60–91 %. Abbreviations: 4-MeO-Ph = 4-methoxyphenyl; 4-N$(CH_3)_2$-Ph = 4-dimethylaminophenyl; 1-Me-5-IND = *N*-methyl-5-indolyl.

methanol (**16**), leaving the secondary sulfonamide unaffected. Selective methylation of the sulfonamide nitrogen, without affecting primary or secondary anilines in the structure, was achieved with methyl iodide in the presence of KOH in dry acetonitrile (**5**, **8**, and **13**). Benzotriazole (**21**) and benzimidazole (**22**) sulfonamides were prepared by cyclization of the *ortho* amino group onto the sulfonamide nitrogen with *tert*-butyl nitrite and ethyl orthoformate, respectively.

Phenstatin and isocombretastatin analogs were synthesized as shown

Table 1
Chemical structure, antiproliferative activity against human tumor cell lines, Tubulin Polymerization Inhibitory activity, and determination of aqueous solubility. Compounds have been divided into four different series according to the substituents on the aromatic A ring or to the bridge between both aromatic rings. [a] Drug concentration required to inhibit the growth of selected human tumor cell lines by 50 %, relative to untreated controls after 72 h of drug exposure. Values are derived from concentration-response curves using the XTT assay as described in the Experimental Section. Data are shown as the mean values of three experiments performed in triplicate. [b] IC_{50} in human colon adenocarcinoma HT-29 cell line in the presence of the Pgp/MDR1 inhibitor Verapamil (10 μM). [c] In vitro Tubulin Polymerization Inhibitory (TPI) IC_{50}. [d] n.d.: not determined. [e] 1-MeIND = *N*-methyl-5-indolyl.

	R	Z	X		Antiproliferative activity IC_{50} (nM)[a]					
				Comp	HeLa	MCF7	HT-29	HT-29 Verap[b]	IC_{50} TPI (μM)[c]	Solub (μg/mL)
Series 1	OCH_3	NH_2	H	**4**	>1000	>1000	>1000	n.d.[d]	>10	243
	OCH_3	NH_2	CH_3	**5**	>1000	>1000	>1000	n.d.	>10	47
	OCH_3	NHCHO	H	**6**	>1000	>1000	>1000	n.d.	>10	16
	OCH_3	$NHCH_3$	H	**7**	411	530	>1000	n.d.	>10	60
	OCH_3	$NHCH_3$	CH_3	**8**	71	71	410	130	32.6	164
	OCH_3	$N(CH_3)_2$	CH_3	**9**	337	390	662	450	>10	71
Series 2	OCH_3	H	H	**10**	>1000	>1000	>1000	n.d.	>10	82
	OCH_3	NH_2	H	**12**	>1000	>1000	>1000	n.d.	>10	460
	OCH_3	NH_2	CH_3	**13**	>1000	>1000	>1000	n.d.	>10	n.d.
	OCH_3	NHCHO	H	**14**	>1000	>1000	>1000	n.d.	>10	n.d.
	OCH_3	$NHCH_3$	H	**15**	>1000	>1000	>1000	n.d.	>10	n.d.
	OCH_3	$N(CH_3)_2$	H	**16**	630	n.d.	>1000	n.d.	>10	30
	OCH_3	NHAc	H	**17a**	>1000	>1000	>1000	n.d.	>10	454
	OCH_3	NHAc	Ac	**17b**	>1000	>1000	>1000	n.d.	>10	480
	OCH_3	NHAc	CH_3	**18**	>1000	>1000	>1000	n.d.	>10	n.d.
	OCH_3	Gly-*t*Boc	H	**19**	>1000	>1000	>1000	n.d.	>10	1172
	OCH_3	Succinic	H	**20**	>1000	>1000	>1000	n.d.	>10	255
	OCH_3	-N=N-N-		**21**	>1000	>1000	>1000	n.d.	>10	220
	OCH_3	-N=CH-N-		**22**	>1000	>1000	>1000	n.d.	>10	4489
Series 3	OCH_3	H	H	**25**	227	350	187	187	>10	13
	OCH_3	NH_2	H	**27**	>1000	>1000	>1000	n.d.	>10	51
	OCH_3	NHCHO	H	**28**	>1000	>1000	>1000	n.d.	>10	44
	OCH_3	$NHCH_3$	H	**29**	697	>1000	>1000	n.d.	>10	189
	OCH_3	$NHCH_3$	CH_3	**30**	1070	>1000	>1000	n.d.	>10	20
	OCH_3	NHAc	H	**31**	>1000	>1000	>1000	n.d.	>10	n.d.
	OCH_3	NCH_3Ac	CH_3	**32**	>1000	>1000	>1000	n.d.	>10	n.d.
	OCH_3	$NHCOCH_2Cl$	H	**33**	>1000	>1000	>1000	n.d.	>10	n.d.
	OCH_3	$NHCO(CH_2)_2Cl$	H	**34**	>1000	>1000	>1000	n.d.	>10	n.d.
	OCH_3	Ethylurea	H	**35**	>1000	>1000	>1000	n.d.	>10	n.d.
	OCH_3	CH_3Ethylurea	CH_3	**36**	>1000	>1000	>1000	n.d.	n.d.	n.d.
Series 4	OCH_3	NH_2	C=O	**40**	283	413	>1000	n.d.	>5	110
	OCH_3	NH_2	$C=CH_2$	**43**	190	65	400	463	2.5	102
	OCH_3	$N(CH_3)_2$	$C=CH_2$	**44**	397	537	2000	600	6.0	n.d.
	OCH_3	NH_2	CH=CH	**48*Z***	190	63	658	613	6.2	n.d.
	OCH_3	$N(CH_3)_2$	CH=CH	**49*Z***	260	57	283	240	1.1	71
	OCH_3	$N^+(CH_3)_3$	CH=CH	**50*Z***	4870	973	>1000	n.d.	>5	1000
	OCH_3	NHAc	CH=CH	**55*Z***	810	677	5500	987	>5	48
	OCH_3	NCH_3Ac	CH=CH	**56*Z***	>1000	>1000	>1000	n.d.	>5	44
	OCH_3	NCH_3Boc	CH=CH	**59*Z***	650	587	>1000	n.d.	>10	n.d.
	OCH_3	$NHCH_3$	CH=CH	**60*Z***	13	4	309	73	0.8	52
	$N(CH_3)_2$	NH_2	CH=CH	**64*Z***	480	753	>1000	n.d.	>5	6.7
B = 1-MeIND [e]		$NHCH_3$	C=O	**67**	13	12	303	430	1.2	10
B = 1-MeIND		NCH_3Boc	$C=CH_2$	**68**	>1000	>1000	>1000	n.d.	>5	6.3
Combretastatin A-4					2	1	305	327	3	1
ABT-751					388	180	213	250	4.4	40

in Scheme 3. Diaryl methanols **38** and **65** were built by nucleophilic addition of aryl-lithium reagents – prepared from the previous treatment of aryl bromides with *n*BuLi – to aldehydes **37** and **58**. Oxidation of the diarylmethanol derivatives with pyridinium dichromate (PDC) gave diarylketones (phenstatins) **39** and **66**. The ketones were then transformed in the 1,1-diarylethenes (isocombretastatins) **42** and **68** by Wittig reaction with triphenylphosphonium methylide. The required phosphonium salts were synthesized from precursor benzylic aldehydes that were reduced to the benzylic alcohols with $NaBH_4$ in methanol (**61**) and then converted into the corresponding benzylic halides (**45** and **62a**) which upon treatment with triphenylphosphine in toluene gave the phosphonium salts **46** and **62b**. Combretastatins (1,2-diarylethenes) (**47**, **55**, **59**, and **63**) were synthetized by Wittig reactions between the phosphonium ylides prepared from phosphonium salts **46** and **62b** with *n*BuLi and benzaldehydes **37**, **54**, and **58**. The *E* and *Z* isomers were chromatographically separated. Diaryl derivatives in series 4 were prepared with NO_2 (**38**, **39**, **42**, **47**, and **63**), NH_2 (**40**, **43**, **48**, and **64**), NHAc (**55**), NCH_3Boc (**59** and **65**), $NHCH_3$ (**60** and **67**), or $N(CH_3)_2$ (**44** and **49**) substituents at *Z* position. Derivatization was performed after diaryl assembly with nitro groups or other substituents in place. Zinc-catalyzed reductions of the nitro groups gave amines **40**, **43**, **48**, and **64**. Methylations of the amines with iodomethane in refluxing acetone gave polymethylated compounds **44**, **49**, and **50**. Preparation of the monomethylated amines was attempted by *N*-^{t}Boc protection and methylation of the monoaryl precursors, synthesis of the diaryl derivatives, and deprotection sequences. Benzaldehydes **54** and **58** were prepared from the nitro precursor **37** before the bridge formation step. First, the aldehyde was protected (**51**) by treatment with ethylene glycol and *p*-toluenesulfonic acid in refluxing toluene, as several attempts of direct reduction of the nitro group led to aldehyde reduction too. Then,

the nitro group was zinc-catalyzed reduced into the corresponding amine (**52**). The protecting group was then removed in methanolic HCl (**53**). Finally, amine derivatization was carried out by acetylation (**54**) or by *N*-^{t}Boc (**57b**) and subsequently methylated to **58**, and the diaryl compounds were synthesized. However, *N*-^{t}Boc deprotection of methylated amides **59** and **66** with p-toluenesulfonic acid in THF failed and was attempted by treatment with trimethylsilyl chloride in methanol. Combretastatin **60** gave only the *E* isomer, and preparations of **67** and **68** were also unsuccessful, with substrate degradation.

2.1.2. Aqueous solubility

One of the aims of the amino groups is to improve the aqueous solubility of the colchicine site ligands (Table 1). The thermodynamic solubility of the synthesized compounds in pH 7.0 phosphate buffer was determined by the shaking flask methodology, consisting of 48 h of shaking followed by microfiltration and spectrophotometric quantification of the compound in solution by the UV absorption measured at the three wavelengths of maximum absorbance for each compound. Combretastatin A-4 and ABT-751 were used as references for comparison, with measured solubilities of 1 μg/mL and 40 μg/mL, respectively. The substitution with amine derivatives on ring A for 4,5-dimethoxyanilines of series 1 and 2 is neatly favorable in terms of solubility for primary amino groups (e.g. compare **10** with **4** and **12**, with a 3 to>5-fold improvement) and acetamides (e.g. compare **10** with **17a**), neutral for methylamino groups (e.g. compare **10** with **7**), and not favorable for formamido groups (e.g. compare **10** with **6**). For series 3 and 4 all these substitutions are favorable. This comparison yields similar results when the replacement of a methoxy group by the amino substituent is considered, as methoxy analogs[10] have similar solubilities as the unsubstituted counterparts. The different behavior of series 1 and 2, and 3 and 4 for changes that imply identical substitutions and therefore in polar surface area (PSA) reflect the complex nature of solvation processes and reinforce the need for exploration of structurally diverse series. Overall, the amino derivatives show quite notable solubility improvements when compared to combretastatin A-4 or ABT-751.

We have calculated other properties which are known to affect the pharmacokinetics of drugs for all the synthesized compounds (Supplemental Table 2). The potent anti-proliferative compounds (see below) **8** and **67** show clogP values lower than 3 or just slightly above 3 for **60*Z***. These values are good predictors of absorption and low non-specific toxicity. Also, compound **8** has a topological polar surface area above the desired 75 Å^2, which is also a good predictor of low toxicity. Compounds **60*Z*** and **67**, although below this threshold show a substantial increase in comparison with the parent non-aminated analogs.

2.2. Biology

2.2.1. Antiproliferative effect

We have assayed by the XTT method the effect of the synthesized compounds on cell viability against three types of human cancer cell lines (Table 1): the highly sensitive to CA-4 HeLa (cervix epithelioid carcinoma), and MCF7 (breast adenocarcinoma), and the CA-4 resistant HT-29 (colon adenocarcinoma). Compounds inhibiting cell proliferation by>40 % at 1 μM in at least three independent assays were evaluated at concentrations in the range between 0.1 nM and 10 μM and half-maximal inhibitory concentrations (IC_{50}) were calculated (Table 1). CA-4 and ABT-751 were used as references, with olefin CA-4 showing nanomolar IC_{50} values against both HeLa and MCF7 and submicromolar IC_{50} values against HT-29 and the sulfonamide ABT-751 showing submicromolar IC_{50} values against the three cell lines, in agreement with previous reports. The three cell lines show similar responses to the compounds, with HT-29 being somewhat less sensitive to the more potent compounds in the series, a difference in sensitivity previously observed for other colchicine site ligands.[22–24].

Methoxybenzene sulfonamides with aminated A rings and a 2,4,5-substitution pattern (series 2 and 3) did not show submicromolar antiproliferative potencies against any of the three cell lines. This is not a necessary consequence of the regiochemical arrangement of the substituents, as we have previously shown that methoxybenzenesulfonamides with 2,4,5-substituted A rings have antiproliferative potencies in the double figures nanomolar range against these cell lines due to tubulin inhibition, especially so when carrying a bromine atom at position 4, which even results in higher potencies than the corresponding compounds with the classical 3,4,5-trimethoxyphenyl A ring or the 4-unsubstituted analogs.[21] The amino substituents at position 4 in series 3 (**27**) cause a potency reduction compared to the unsubstituted analog **25**. This is unlikely due to steric hindrance, as active 4-methoxy or 4-bromo substituents are similar in size to the amino substitutions, such as the *N*-methylamino in compounds **29** and **30**. Intriguingly, DFT calculations (see below) of the preferred conformations (Supplemental figure SF1) for the more potent (Table 1) 5-methoxy group with *ortho* bromine or secondary amido substituent (e.g. **28** and **31**) place the methyl carbon in the ring planes, whereas amines such as those in **29** and **30** form intramolecular hydrogen bonds to the methoxyl oxygen (secondary amides do it as well) but displace the methyl out of the ring plane, thus providing a possible explanation for the observed potency loss, either caused by steric hindrance or a geometrical rearrangement that reduces the hydrogen bond quality. For the amides, the additional carbonyl functionality seems detrimental to the binding.

Methoxybenzene sulfonamides with aminated A rings and a 3,4,5-substitution pattern (series 1) only show sub-micromolar anti-proliferative potencies against any of the three cell lines for the methylamino substituents **7** and **8**, and dimethylamino **9**. **8** shows higher potency than ABT-751 but lower than CA-4, while **7** and **9** show potencies similar to ABT-751. In this case, intramolecular hydrogen bonds between the amine hydrogen (acting as the donor) and the methoxyl oxygen (acting as the acceptor) force again the 4-methoxy carbon out of the ring plane. This is reminiscent of the central methoxy group of the 3,4,5-trimethoxyphenyl ring where steric hindrance and the repulsions between oxygen lone pairs place the central methyl out of the ring plane and set the oxygen lone pair in a good geometrical arrangement for interacting with the thiol group of Cys-241β[25] of tubulin, an interaction deemed to govern the interaction with classical colchicine site ligands carrying such a moiety. The primary anilines **4** and **5** are devoid of anti-proliferative activity, probably reflecting their unfavorable desolvation against the hydrophobic residues of the trimethoxyphenyl ring binding sub-pocket of tubulin. Formamide **6** has a similar geometry as methylamine **8**, but it shows no anti-proliferative effect, probably caused by unfavorable interactions with the tubulin sidewalls. Methylamines **7** and **8** show a very similar geometry and contact surface with the tubulin walls as the 3,4,5-trimethoxyphenyl ring, thus explaining the high anti-proliferative potencies. As previously observed, methylation of the sulfonamide nitrogen results in a higher potency.[10,21,26] The high potency and improved water solubility of methylamine **8** suggest its involvement as a masked polar group (MPG),[27] switching from a solvent-exposed NH bond in water that helps with the solubility to a masked one by intramolecular hydrogen bonding to the nearby methoxy group oxygen in the highly hydrophobic colchicine site.

Combretastatins, phenstatins, and isocombretastatins with amino groups (series 4) show consistently anti-proliferative potencies below 0.5 μM, while the amides (**55*Z***, **56*Z***, **59*Z***, and **68**) and the ammonium salt (**50*Z***) show micromolar or at best higher than 0.5 μM potencies. Again, a potency order of MeNH- > $-NH_2$ >~ Me_2N- is in good agreement with the tubulin interaction pattern observed in the sulfonamide series (Supplemental Figure SF1). Interestingly, a higher sensitivity (differences in IC_{50} values>3-fold) is observed for MCF7 than for HeLa cells for combretastatins **48*Z***, **49*Z***, and **60*Z***, and isocombretastatin **43**, all of them amongst the most soluble of the series due to the polar amino substituents. Different interactions with tubulin seem unlikely to be the reason, as they all share the interaction patterns with sulfonamides with similar potencies against HeLa and MCF7. Therefore, a different cellular

uptake might account for the observed differences. The reduced fold difference in potency between HeLa and the resistant HT-29 suggests a reduced uptake in HeLa cells of the polar compounds[28]. Again, the masked polar group incorporation (MPGI) strategy seems to be in effect for the most potent compounds (e.g. **60*Z*** and **67**).

2.2.2. Tubulin polymerization inhibition (TPI)

We have measured by turbidimetry the effect of the compounds on the polymer mass formed from bovine brain tubulin under in vitro assembly conditions (Table 1). Compounds inhibiting tubulin polymerization by>40 % at 10 μM were assayed at a range of concentrations to determine the IC_{50} values of inhibition of microtubular protein polymerization (TPI). These values allowed us to compare the inhibitory potency on the polymerization of tubulin of the compounds and to the reference compounds combretastatin A-4 (one of the most potent TPI with IC_{50} values between 1 and 3 μM) and ABT-751 (a sulfonamide with a TPI IC_{50} value of 2–4 μM), and to analyze the structure-TPI relationship.

The TPI results follow similar trends to the cytotoxicity results, with the most cytotoxic compounds showing potent TPI, thus suggesting an implication of tubulin polymerization inhibition in the action mechanism. As previously observed, sulfonamides show substantially higher TPI values (i.e. are less potent) than combretastatins, isocombretastatins, or phenstatins for similar cytotoxicity values (e.g. compare sulfonamide **8** with compounds of series 4), an effect that seems to depend on the scaffold.[10,21,26] This discrepancy has been proposed to arise by an effect associated more with an alteration of polymerization dynamics causing the cytotoxicity at low drug concentrations (in the nanomolar range) than with changes of polymer mass as seen in the TPI assays.[27].

The most potent compounds in the TPI assay are methylanilines **60*Z*** (0.8 μM) and **67** (1.2 μM), dimethylaniline **49*Z*** (1.1 μM), and unsubstituted aniline **43** (2.5 μM), all of them more potent inhibitors than CA-4 (3 μM). The potency order observed in the cytotoxic potencies for the amino substituents (MeNH- > $-NH_2 \geq Me_2N$-) is preserved here for the first two, but the dimethylanilines seem to be more potent in this assay and therefore suggest that their lower potency is not related to reduced interaction with tubulin. Due to steric hindrance, the *ortho*-substituted dimethylanilines are less planar and their nitrogen lone pair is less conjugated to the phenyl rings than the corresponding methyl and unsubstituted anilines. Therefore they are expected to be more basic and might be protonated and therefore more polar, in good agreement with the similar behavior in the selectivity against MCF7 vs HeLa cells suggested to arise from a higher polarity (see above) for compound **49*Z*** and the other compounds in the group (i.e. **48*Z***, **60*Z***, and **43**) which carry a proton on the amine substituent.

2.2.3. Effects on cellular microtubules

The effects on the cellular microtubules have been shown to provide a much better correlation to the cytotoxicity for microtubule destabilizing drugs than the TPI, and therefore provide good support for the tubulin inhibitory mechanism proposal. We have studied by immunofluorescent confocal microscopy the effects on the microtubule networks of HeLa and MCF7 cells of sulfonamide **8**, combretastatin **60*Z***, and phenstatin **67** (Fig. 1), the most potent compounds of their respective series belonging to different scaffolds and showing different TPI and cytotoxicity potencies, with **8** being less potent than **60*Z***, and **67**, the most potent compounds in both assays.

The three compounds promote a drastic and severe disruption of the microtubule network in HeLa cells. Compound **60*Z*** showed a similar disruption of the microtubule network in MCF7 cells as it does in HeLa cells. **8** and **67** disrupted to a lesser extent the microtubule network of MCF7 cells, whose microtubules had lost the hairy appearance of the untreated controls. MCF7 cells treated with the three compounds showed multi-lobulated nuclei consistent with mitotic disruption and further support the proposal that the compounds act by tubulin inhibition.

2.2.4. Sensitivity to MDR transporters

The compounds showed significantly lower IC_{50} values against HeLa and MCF7 than against HT-29 cells, which turned out to be the less sensitive cell line assayed, as previously observed for related colchicine site inhibitors. Several mechanisms have been proposed for the low intrinsic sensitivity of HT-29 cells to CA-4, including autophagy,[22] MDR efflux[23], and inactivation by metabolic transformation by UDP-glucuronidation.[9] The structures of the synthesized compounds are not compatible with glucuronidation reactions, so we have studied their possible sensitivity to efflux by MDR proteins by pharmacological co-treatment with the MDR inhibitor verapamil and comparison of the IC_{50} values in the absence and in the presence of the inhibitor in a concentration that has been shown not to affect cell proliferation. We have selected a threefold or larger reduction in the IC_{50} cytotoxicity values as the threshold for considering the compounds MDR-efflux susceptible. According to this cutoff, combretastatin A-4 would not be

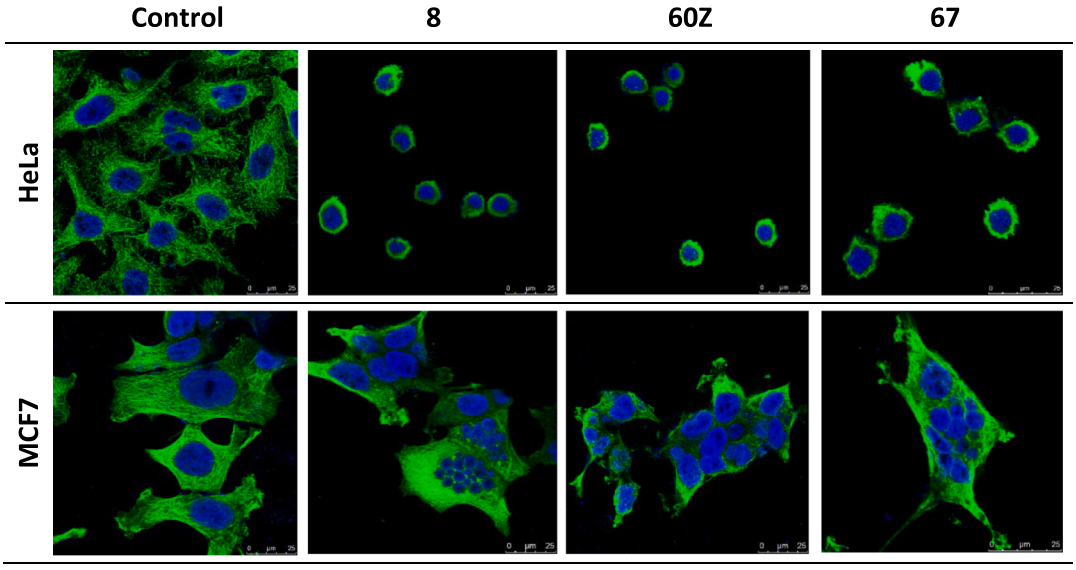


Fig. 1. Effects of the treatment with compounds **8** (1 μM), **60Z** (50 nM), or **67** (50 nM) on the microtubule network in HeLa and MCF7 cells by confocal microscopy. α-Tubulin was stained in green, and nuclei were stained in blue. Scale bar: 25 μm.

a substrate for MDR efflux and neither would ABT-751. In good agreement, the resistance of HT-29 cells to combretastatin A-4 has been recently assigned to mechanisms other than MDR,[9,22] and ABT-751 is known to not be a substrate for the MDR pump.[29] According to this threshold, for the compounds with submicromolar potencies against HeLa and MCF7 only **8**, **44**, and **60*Z*** would be MDR substrates, and therefore the lower sensitivity observed against HT-29 for the rest must have a different origin. **8**, and **60*Z*** are methylanilines, but methylaniline **67** is not a substrate.

2.2.5. Effects on the cell cycle and induction of apoptosis

We have studied the effects of compounds **8**, **60*Z***, and **67** on the cell cycle of HeLa (Fig. 2a), and MCF7 (Supplemental Fig. 2) cancer cell lines and the non-tumorigenic cell line HEK-293 (Supplemental Fig. 2) after different treatment times (24, 48, and 72 h). The compounds arrest the cell cycle of HeLa and MCF7 at G_2/M. For HEK-293, **8** arrests the cell cycle at G_2/M, but instead **60*Z***, and **67** accumulate cells with a $SubG_0/G_1$ DNA content, indicative of apoptotic induction (Fig. 2b). An overtime increase of cells with $SubG_0/G_1$ DNA content is observed, which is delayed in MCF7 cells compared to HeLa. For compound **8**, this increase is reduced in the non-tumorigenic cell line HEK-293, whereas for **60*Z***, and **67** the accumulation of apoptotic cells is larger in HeLa, next in HEK-293 cells, and the least in MCF7 cells.

The time-course analyses showed different evolution of the cell cycle distribution profiles of the different cell lines for the compounds (Fig. 4). Sulfonamide **8** at 1 μM after 24 h of incubation had already arrested most of the HeLa, MCF7, and HEK-293 cells at G_2/M (60.1 %, 33 %, and 44.8 %, respectively) and started to accumulate cells with $SubG_0/G_1$ DNA content indicative of apoptotic induction (19.9 %, 9.1 %, and 9.6 %, for HeLa, MCF7, and HEK-293 cells respectively compared to 1.2 %, 3.7 % and 4.8 % for their respective untreated controls). After 24 h of treatment with 50 nM combretastatin **60*Z***, the HeLa and MCF7 cells accumulated at G_2/M (44.9 %, and 56.9 %, respectively), whereas the HEK-293 cells had a reduced G_2/M population (9.6 %, compared to 25.6 % of the untreated control) and an enlarged population of cells with $SubG_0/G_1$ DNA content (25.8 %, compared to 4.8 % of the untreated control), which is also observed for HeLa cells (33.1 %, compared to 1.2 % of the untreated control). Phenstatin **67** at 50 nM showed a similar result to that of combretastatin **60*Z*** for the G_2/M accumulation in HeLa, and MCF7 cells (63.5 %, and 61.6 %, respectively compared to their controls at 27.7 %, and 17 %, respectively) and in the maintenance of low levels in HEK-293 cells (7 %, compared to 25.6 % of the untreated control). It differed from combretastatin **60*Z*** in the evolution of the cell population with $SubG_0/G_1$ DNA content just for MCF7 cells, as it similarly increased in HeLa (16.3 %, compared to 1.2 % of the untreated control) and HEK-293 cells (21.8 %, compared to 4.8 % of the untreated control) but was unchanged for MCF7 cells (0.6 %, compared to 3.7 % of the untreated control).

After 48 h of treatment, sulfonamide **8** continued the G_2/M arrest of HeLa, MCF7, and HEK-293 cells (57.8 %, 34.3 %, and 40.7 %, respectively) and slightly increased or maintained the percentage of cells with $SubG_0/G_1$ DNA content (25 %, 14.9 %, and 8.9 %, respectively) compared to the 24 h time point. Combretastatin **60*Z*** and phenstatin **67** caused a similar effect on the G_2/M arrest of the HeLa and MCF7 cells (56.8 %, and 50.9 %, respectively for **60*Z***, and 55.9 %, and 68.1 %, respectively for **67**), whereas HEK-293 cells recovered a G_2/M population similar to the untreated control (20.6 % for **60*Z***, and 23.6 % for **67** compared to 22.3 % of the untreated control). They maintained the enlarged population of cells with $SubG_0/G_1$ DNA content in HeLa cells (24.9 % for **60*Z***, and 26 % for **67** compared to 1.1 % of the untreated control) and started to increase it in MCF7 cells (8 % for **60*Z***, and 3.4 % for **67** compared to 5.3 % of the untreated control).

At the final time point of 72 h, corresponding to the cytotoxicity measurements, sulfonamide **8** reduced the G_2/M population from the previous time points in HeLa and HEK-293 cells (37.3 % and 33.6 % respectively), while it further increased in MCF7 cells (44.5 %). These changes in the G_2/M populations come with significant increases in the $SubG_0/G_1$ cell populations for the three cell lines (increases of 23.7 %, 11.6 %, and 8.4 % from the 48 h values, respectively). Phenstatin **67** and combretastatin **60*Z***, again showed similar effects on the cell cycle populations at 72 h. HeLa and MCF7 reduced the G_2/M accumulation (by 26.4 % and 15.7 % respectively for **60*Z*** and by 12.7 % and 19.9 % respectively for **67**), whereas HEK-293 cells maintained the G_2/M populations (27.7 % and 16 % respectively). This dual behavior also applies to the cells with $SubG_0/G_1$ DNA content, with large enlargements for HeLa and MCF7 compared to the 48 h (30.2 % and 4.3 % increases respectively for **60Z** and 19.3 % and 5 % respectively for **67**), while for HEK-293 a similar level of cells is seen for each of the three time-points (25.8–27.4–29.3 % for **60*Z***, and 21.8–15.3–24.4 % for **67**).

In summary, compounds **8**, **60*Z***, and **67** arrest the cell cycles of HeLa cells at G_2/M from early time points, and they are reduced over time accompanied by an increase in the populations with $SubG_0/G_1$ DNA content (the sum of the two populations is maintained throughout at values around 82 % for the three treatments), which peaked at 72 h. For MCF7, the accumulated populations are roughly maintained for the first two time-points with a higher contribution of $SubG_0/G_1$ DNA content cells for **8**. At the 72 h time-point, **8** reaches similar contributions of both populations but the other two compounds cause an overall reduction due to a decrease in the G_2/M population. HEK-293 cells show different rates of accumulation in the combined populations depending on the compounds: **8** rapidly reaches a plateau around 50 % with increased $SubG_0/G_1$ DNA content cells at 72 h at the expense of the G_2/M population, whereas **60*Z***, and **67** show a slower overtime increase of the accumulated populations due to modest increases in the G_2/M populations as the population of cells with $SubG_0/G_1$ DNA content remained roughly invariant at around 25 % and 20 %.

The confocal immunofluorescence experiments show a disruption of the microtubule network and the cell cycle profiles are a strong indication that the synthesized compounds are acting as antimitotic drugs in HeLa cells. Therefore, we have studied the appearance of the mitotic marker MPM-2 upon treatment with the compounds. Increased detection of MPM-2 signal is observed by Western blot 24 h after treatment (Fig. 3) which subsequently declines at 48 h (data not shown), in very good agreement with the observed cell cycle profiles.

To better ascertain the apoptotic cell death suggested by the cell cycle studies, we analyzed by dual channel flow cytometry HeLa, MCF7, and HEK-293 cells doubly stained with Annexin V-FITC (AnV) and propidium iodide (PI) after 72 h treatment with compounds **8**, **60*Z***, and **67** (Fig. 4). Doubly negative (AnV–/PI–) cells are considered alive, AnV+/PI– in Early Apoptosis (EA), double-positive (AnV+/ PI +) in Late Apoptosis (LA), and AnV–/PI + in necrosis (Table 2). Alive control cells were always > 88 %. Consistently with the cell cycle results, a strong apoptotic response was observed for the HeLa cells, which had an almost complete apoptotic profile (>80 %). On the opposite end, treated MCF7 cells showed similar levels of apoptotic cells as the untreated control, around 10 %. A weak build-up of the apoptotic response in MCF7 cells could potentially be explained by their deficiency of caspase 3,[30] the hub effector of the apoptotic machinery. HEK-293 cells show elevated levels of late apoptotic cells (13 to 27 %, compared to 5 % of the control), in agreement with the early appearance of the population with $subG_0/G_1$ DNA content which does not increase over time (the correspondence of the respective populations in the two experiments is very good). These results are thus consistent with the cell cycle experiments and support the interpretation that compounds **8**, **60*Z***, and **67** cause mitotic arrests that trigger the apoptotic response responsible for the antitumor effect, as has been previously described for other antimitotic agents.[31] Furthermore, the lower apoptosis induction of the compounds on HEK-293 cells compared to HeLa suggest that the compounds might have stronger effects against tumorigenic than non-tumorigenic cells, which might provide selectivity. For MCF7 cells, the slower start of the apoptotic response observed here does not imply necessarily lower antitumor effects, as it has been observed that apoptosis

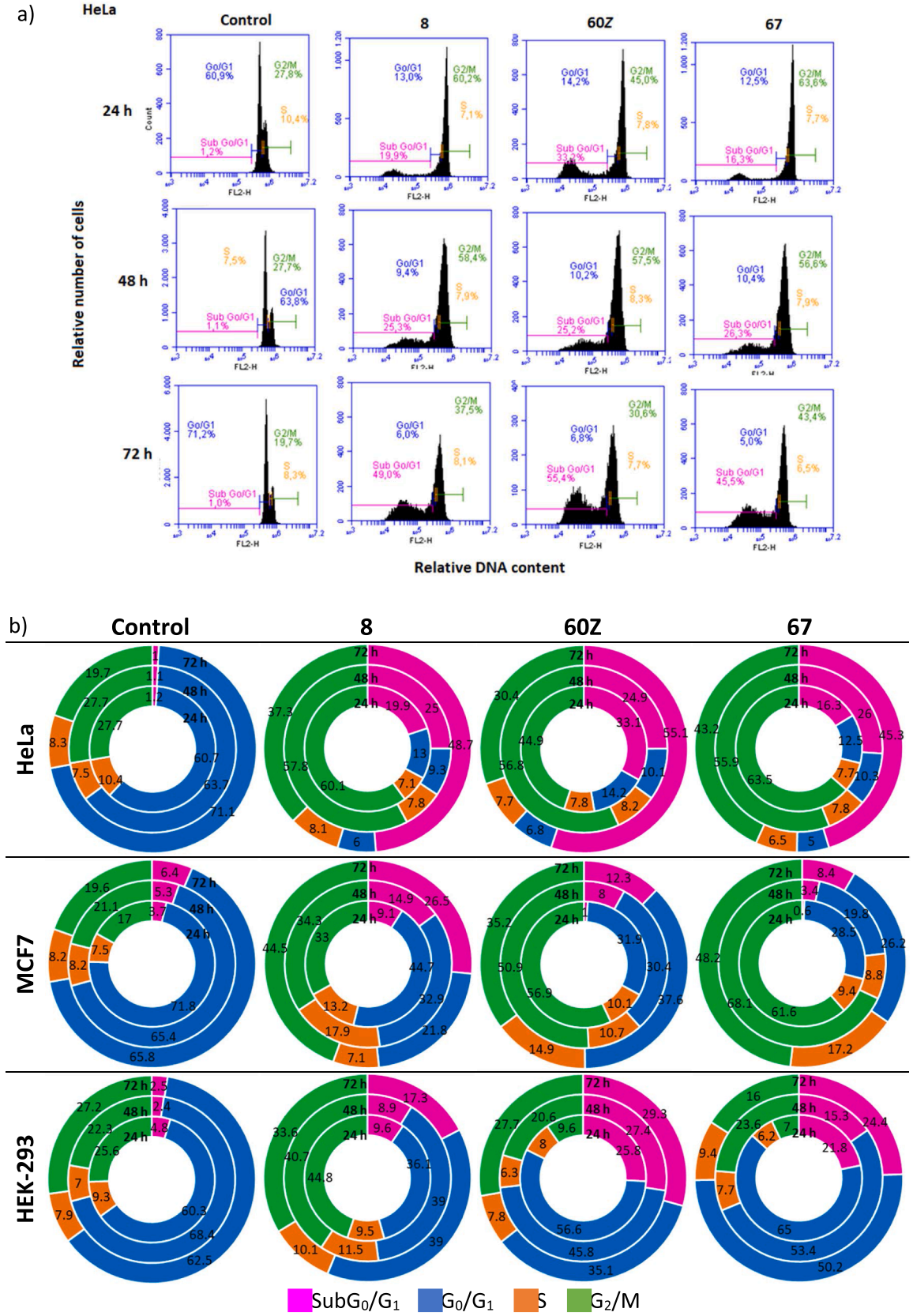


Fig. 2. A: histograms of the cell cycle of hela cells after 24, 48, or 72 h of treatment with compounds **8** (1 μM), **60Z** (50 nM), or **67** (50 nM). Untreated control cells were run in parallel. b: Time course analysis of the effect of compounds **8** (1 μM), **60Z** (50 nM), and **67** (50 nM) on the subG$_0$/G$_1$, G$_0$/G$_1$, S, and G$_2$/M cell cycle phases in HeLa, MCF7, and HEK-293 cells. Cells were incubated for 24, 48, and 72 h, stained with propidium iodide, and then their DNA content was analyzed by flow cytometry as described in the Experimental Section. The different cell cycle phases were quantified, expressed in percentages, and represented in concentric hollow circles (24 h inner, 48 h center, and 72 h outer). Untreated control cells were run in parallel.

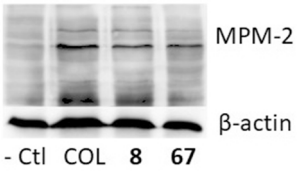


Fig. 3. Western blot analysis of the levels of the mitotic marker MPM-2 in HeLa cells after treatment with **8** at 1 μM and **67** at 100 nM. Untreated controls (-Ctl) and treatment with Colchicine (COL) at 100 nM are included for comparison.

eventually develops in these cells upon treatment with antimitotic drugs in a process where caspases 6 or 7 replace caspase 3.[30].

2.3. Computational studies

2.3.1. Conformational analysis of the A ring modifications

The chemical modifications on colchicine site scaffolds here described, that is, the substitution of a hydrogen atom of a 4,5-dimethoxyphenyl A ring by an amino (unsubstituted, methylamino, dimethylamino, formamido, or acetamido) substituent at different positions, are expected to have important electronic and conformational consequences with respect to the classical 3,4,5-trimethoxyphenyl ring of colchicine site ligands, that affect their binding to the colchicine site of tubulin. To this end, we DFT calculated the preferred conformations for every synthesized modification and compared them with those of a 3,4,5-trimethoxyphenyl ring (both in the minimum energy and in the conformations found in the X-ray structures with tubulin) to rational their effects on the found biological activities of the compounds that carry them (Supplemental Figure SF1).

The 3,4,5-trimethoxyphenyl rings of colchicine site ligands bound to tubulin adopt conformations with the central methoxy group out of the phenyl ring plane due to steric hindrance and directing one of its electron lone pairs towards the thiol group of Cys241β of Helix H7, while the methyls of the two lateral methoxy groups are within the ring plane, giving to the whole moiety a quite planar disposition. This same disposition is the minimal energy conformation found in the DFT model. For 4,5-dimethoxyphenyl analogs with a 3-amino-, 3-methylamino-, 3-formamido-, and 3-acetamido- substituents, intramolecular hydrogen bonds form between the N—H and the 4-methoxy oxygen. This places the other substituent of the nitrogen within the ring plane, forces the central 4-methoxy out of the plane, and the 5-methoxy stands as described for the 3,4,5-trimethoxyphenyl ring, thus imposing a high structural similarity to this moiety. In water, the outwards rotation of the NH exposes the polar groups and increases the polarity and aqueous solubility, thus behaving as a masked polar group (MPGI strategy). For di-substituted anilines, steric hindrance forces the *N* substituent out of the ring plane and distorts the planar shape of the trimethoxyphenyl ring observed in the tubulin complexes.

2.3.2. Docking studies

We have investigated the binding mode of every synthesized compound (Fig. 4) at the colchicine site of tubulin using ensemble docking. We used in the docking experiments 46 X-ray structures of tubulin in complexes with colchicine site ligands and 6 structures from molecular dynamics simulations obtained as previously described[18,33], 52 models that represent different arrangements of pharmacophoric points and efficiently sample the receptor flexibility.[32]. We docked the

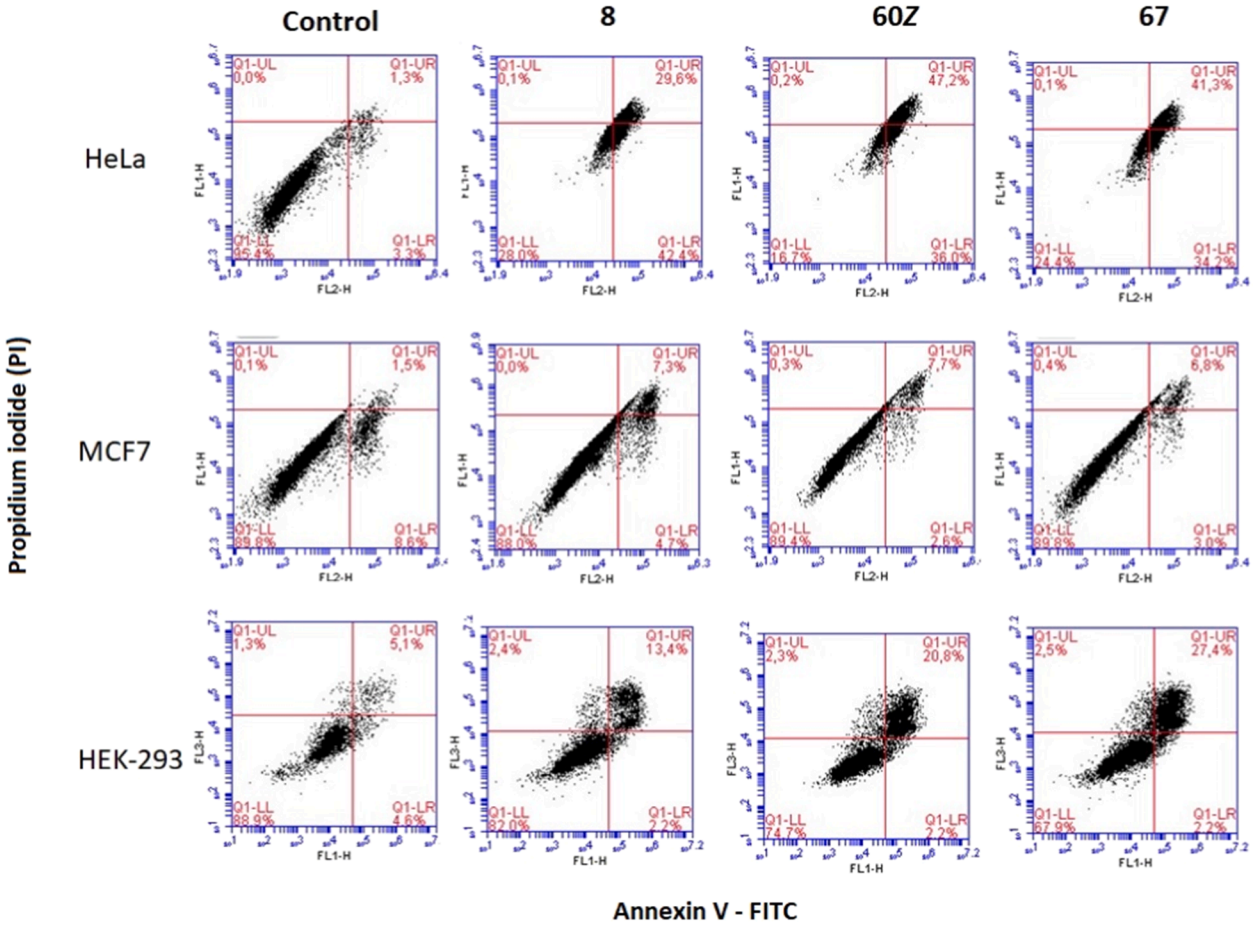


Fig. 4a. Annexin V-FITC and propidium iodide (PI) profiles in HeLa, MCF7, and HEK 293 cells 72 h after treatment with compounds **8** (1 μM), **60Z** (50 nM), or **67** (50 nM). Untreated control cells were run in parallel.

Table 2
Annexin V-FITC and propidium iodide (PI) profiles in HeLa, MCF7, and HEK-293 cells 72 h after treatment with compounds **8** (1 μM), **60Z** (50 nM), or **67** (50 nM). Untreated control cells were run in parallel. Cells were classified as double-negatives (alive cells), AnV-positive cells (early apoptosis), double-positives (late apoptosis), and PI-positive cells (necrosis).

		Annexin V-FITC/PI 72 h (%)			
		Live	Early apoptosis	Late apoptosis	Necrosis
HeLa	Control	95.4	3.3	1.3	0
	8	28	42.4	29.6	0
	60Z	16.7	36	47.2	0.1
	67	24.4	34.2	41.3	0.1
MCF7	Control	89.8	8.6	1.5	0.1
	8	88	4.7	7.3	0
	60Z	89.4	2.6	7.7	0.3
	67	89.8	3	6.8	0.4
HEK-293	Control	88.9	4.6	5.1	1.3
	8	82	2.2	13.4	2.4
	60Z	74.7	2.2	20.8	2.3
	67	67.9	2.2	27.4	2.5

virtual compounds with two software that use very different scoring functions (PLANTS [34] and AutoDock 4.2 [35]). The analysis of the docking results applied a fully automated consensus strategy for the selection of the binding poses based on geometry and scoring comparison. The automatic assignment of the occupancy of the colchicine site subpockets for each ligand pose was done by geometric comparison with references binding in known dispositions: a) the calculated RMSD between every ligand pose and diphenyl scaffolds with the same bridge as the ligand placing the phenyl rings at the A, B, or C sites of the colchicine site of tubulin; b) the RMSD calculations of the deemed common atoms of the poses with those of combretastatin A-4 (for A-B binding), MI-181 (for A-C binding), or ABT-751 (for A-B-C binding) in their X-ray structures (pdb[36] IDs 5LYJ, 4YJ2 and 3HKC, respectively) in complex with tubulin; and c) the pocket occupancy for each ligand calculated by measuring the lowest distances of the poses to a central point of each sub-pocket, as determined by the pharmacophores derived from the X-ray structures of colchicine site ligands in complex with tubulin. According to these calculations, occupied binding sub-pockets were assigned to each pose. Subsequently, the binding modes with the best combined scoring from the two software were selected as the proposed binding mode. To make the comparisons of the scoring values proportionate, Z scores were calculated for each ligand and docking program and the poses ranked between 0 (worse) and 1 (best). We have applied this procedure for colchicine site ligands with known X-ray structures when in complex with tubulin and the procedure retrieves the correct poses in most cases, only failing with ligands with>7 rotatable bonds which do not properly converge in coherent structural superimpositions.

For all the compounds (Fig. 4) the amino substituted 3,4-dimethoxyphenyl rings occupy the A zone, a hydrophobic pocket formed between helices H7 and H8, sheets S8, S9, and S10, and the T7 loop. The A subpocket contacts the methoxylated rim with the sidechains of Ala316β (S8), Ile318β (S9), Lys352β (S9), Ala354β (S9), and Ile378β (S10), above and underneath the ring plane with Leu248β (T7), Ala250β (H7), Cys241β (H7), and Ile378β (S9) on the outer side towards the interdimer surface, and Leu255β (S9) on the inner side deep in the β subunit. The thiol group of Cys241β (H7) sits close to the oxygen lone pair of the 4-methoxy group, which protrudes out of the ring plane as shown in the DFT calculations and the X-ray structures, a disposition that places the oxygen lone pair towards the thiol group. The B rings are placed between helix H8 (above the ring plane) and sheets S8 and S9 (behind the ring plane), making hydrophobic contacts with Met259β (H8), Thr314β (S8), and Lys352β (S9), and with the side chain of Asn258β (H8) contacting with the ring plane. The bridges open toward the αβ interface.

The amino substituents adopt conformations compatible with the DFT calculated more stable conformations. The methylamino substituents preferentially place the methyl groups towards the walls of the A site and the NH vector points towards the neighbor methoxy group in a geometry consistent with an intramolecular hydrogen bond. For unsubstituted amino substituents, the phenyl ring is rotated to place the second NH vector towards the more polar C zone. Dimethylamino substituents are placed with the methyl groups protruding out of the ring plane, in agreement with the DFT calculations. This extra volume generates steric collisions with the pocket walls, forcing the ring to rotate to accommodate it, in agreement with the lower observed potency in the biological assays. Concerning the scaffolds, compounds with one-atom bridges (isocombretastatins and phenstatins) are more rigid than those with two-atom bridges (sulfonamides and combretastatins), which thus overlap better their phenyl rings with those of combretastatin A-4. Additionally, the wider angle and lower displacement of the phenyl rings centroids of phenstatins compared to the two atoms bridged compounds place the lateral substituent closer to the A pocket walls so that bulkier substituents must be allocated towards the groove connecting to the C zone.

Usually, the pair of consensus poses for each ligand belongs to complexes with different protein models. This is probably a result of the different scoring functions selecting for different protein configurations and ligand interactions. Furthermore, not all the best-scored poses for each program belong to the same protein model, although some clustering is observed (for AD4 the most populated protein models are 6BRY and 5XAG,[36] the first with the three most potent ligands of series 4 and the second with the most potent sulfonamides, and the most populated ones for PLANTS are 5LYJ and 5GON,[36] the first including the most potent sulfonamides and the second the most potent

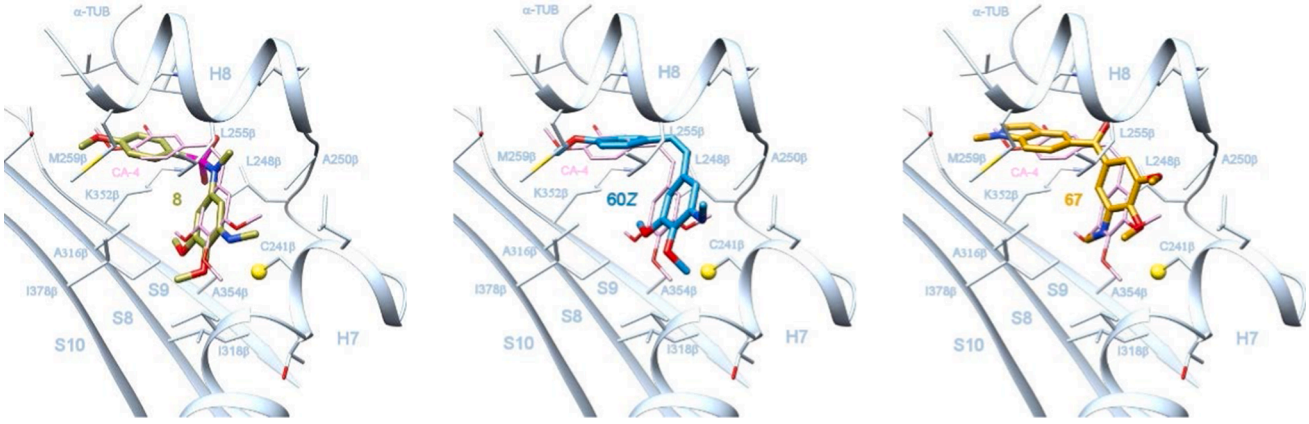


Fig. 4b. Docking poses for compounds **8** (olive), **60Z** (dodger blue) or **67** (orange) in complex with tubulin (5LYJ). Tubulin is in powder blue. The X-ray structure of CA-4 (thin pink bonds) as in 5LYJ is superimposed for comparison.

compounds of series 4). These results reinforce the strategy of applying ensemble docking strategies and validate the consensus scoring approach followed. Unfortunately, the global scoring results using all the protein models do not provide a good correlation with the biological results obtained. Considering that the consensus poses for the most potent compounds cluster in few protein models and that this clustering varies depending on the ligand scaffolds, we have analyzed in isolation the scoring results for every protein model of the main aforementioned clusters (two per program by two scaffolds –series 1 to 3 and 4- for a total of 4 possibilities) and we have compared the ordering of the scorings to the biological results. For the sulfonamides (series 1 to 3), Autodock scoring outperforms PLANTS, with the XAG model providing the best performance. On the other hand, for series 4 PLANTS scoring outperforms Autodock scoring, with slightly better results for the 5GON model than for the 5LYJ one.

3. Conclusions

The low aqueous solubility of colchicine site tubulin inhibitors is one of the main drawbacks in their clinical development. The introduction of amino groups on the B ring has proven itself a successful strategy, but the introduction of amino groups on the A ring (typically a 3,4,5-trimethoxyphenyl) has remained largely unexplored. We have designed and synthesized new colchicine site ligands carrying amino substituents on the A ring in the context of different scaffolds and A ring substitution patterns. The amino-substituted analogs and particularly the methylamino ones are potent tubulin polymerization inhibitors and cytotoxic compounds against several human cancer cell lines while significantly improving the intrinsic aqueous solubility. The compounds are mostly not substrates of the MDR pump, one of the main resistance mechanisms of cancer cells in the clinic. In agreement with the tubulin inhibitory activity shown, the compounds cause an early mitotic arrest in human cancer cell lines that is followed by an apoptotic response, whose extent is kept at lower values for the non-tumorigenic cell line HEK-293, thus suggesting a certain degree of selectivity towards cancer cells. Molecular modeling studies support binding at the colchicine site with the anilines located at the A ring subpocket while hiding the polar hydrogen on the amino groups when present through intramolecular hydrogen bonds to their neighboring methoxyl oxygens. These hiding in apolar environments (tubulin) of polar groups while exposing them in polar solvents (water) to improve the aqueous solubility represents a new successful application of the masked polar group incorporation (MPGI) strategy towards new colchicine site antimitotic agents with improved solubility and with favorable calculated clogP and TPSA values, deemed as predictors of good absorption and low toxicity, as well. The methylamino substituted compounds combine a nanomolar cytotoxic potency with improved water solubilities and are promising candidates for further studies. The introduction of amino substituents on the A ring of colchicine site ligands is a structural modification that could be applied to improve colchicine site ligands with low solubility.

4. Experimental section

4.1. Chemistry

4.1.1. General chemical techniques

Reagents were used as purchased without further purification. Solvents (EtOAc, DMF, CH_2Cl_2, MeOH, CH_3CN, toluene, 2-propanol, and benzene) were stored over molecular sieves. THF was refluxed with sodium/benzophenone, and hexane was dried by distillation and stored over $CaCl_2$. TLC was performed on precoated silica gel polyester plates (0.25 mm thickness) with a UV fluorescence indicator 254 (Polychrom SI F254). Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063; Merck) chromatography. Melting points were determined on a Buchi 510 apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$, CD_3OD, DMSO-D6, or acetone-D6 on a Bruker WP 200-SY spectrometer operating at 200/50 MHz, or a Bruker SY spectrometer at 400/100 MHz. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane and coupling constants (*J* values) are given in Hz. IR spectra in KBr disk were run on a Nicolet Impact 410 Spectrophotometer. A hybrid QSTAR XL quadrupole/time of flight spectrometer was used for HRMS analyses. GC–MS spectra were performed using a Hewlett-Packard 5890 series II mass detector. A Helios-α UV-320 from Thermo-Spectronic was used for UV spectra.

4.1.2. Chemical synthesis

2-Methoxy-4,6-dinitrophenol (1). To a stirred solution at 0 °C of 2-methoxyphenol (5 g, 4.52 mL, 40.3 mmol) in acetic acid (60 mL), nitric acid (5.54 mL, 80.6 mmol) in acetic acid (40 mL) was slowly added under nitrogen atmosphere. After 6 h at 0 °C, the reaction mixture was poured onto ice and kept at 4 °C for 1 h. Then, the precipitate formed was filtered and washed with water under vacuum to dryness to obtain 7.77 g (36.6 mmol, 90 %) of **1**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.06 (3H, *s*), 7.95 (1H, *d*, *J* = 2.8), 8.67 (1H, *d*, *J* = 2.8), 11.18 (1H, *bs*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 57.3 (CH_3), 111.0 (CH), 112.7 (CH), 139.3 (2C), 150.6 (C), 150.9 (C). HRMS ($C_7H_6N_2O_6$ + H^+): calcd 215.0299 (M + H^+), found 215.0299.

1,2-Dimethoxy-3,5-dinitrobenzene (2). To a solution of **1** (5.27 g, 24.6 mmol) and K_2CO_3 (11.9 g, 86 mmol) in acetone (100 mL), $(CH_3)_2SO_4$ (11.7 mL, 123 mmol) was dropwise added. The reaction mixture was stirred at room temperature for 48 h and then heated at reflux for 8 h. The mixture was filtered through Celite© and evaporated to dryness to afford compound **2** (5.52 g, 24.2 mmol, 98 %). The product was then used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.03 (3H, *s*), 4.09 (3H, *s*), 7.95 (1H, *d*, *J* = 2.8), 8.25 (1H, *d*, *J* = 2.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 57.0 (CH_3), 62.4 (CH_3), 110.2 (CH), 112.1 (CH), 142.4 (C), 143.8 (C), 148.0 (C), 154.3 (C). HRMS ($C_8H_8N_2O_6$ + H^+): calcd 229.0455 (M + H^+), found 229.0470.

4,5-Dimethoxybenzene-1,3-diamine (3). A mixture of **2** (5.48 g, 24 mmol), 20 mg of Pd (C), and 125 mL of EtOAc was maintained under low hydrogen pressure and stirred at room temperature. After 4 days, uptake of H_2 was completed and the solution was filtered through Celite. The filtrate was concentrated in vacuo to give product **3** (3.32 g, 19.7 mmol, 82 %). The product was then used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.76 (3H, *s*), 3.78 (3H, *s*), 5.72 (1H, *d*, *J* = 2.8), 5.74 (1H, *d*, *J* = 2.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.5 (CH_3), 60.1 (CH_3), 90.6 (CH), 95.1 (CH), 140.9 (C), 143.2 (C), 147.0 (C), 153.5 (C). GC–MS ($C_8H_{12}N_2O_2^+$): 168 (M^+).

***N*-(3-amino-4,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (4).** To a solution of **3** (2.82 g, 16.7 mmol) in CH_2Cl_2 (100 mL) and pyridine (2.7 mL, 35.5 mmol), 4-methoxybenzenesulfonyl chloride (865 mg, 8.38 mmol) was slowly added. After complete uptake of 4-methoxybenzenesulfonyl chloride, another 865 mg (8.35 mmol) were slowly added. The reaction mixture was stirred at room temperature for 4 h. Then, it was washed with brine, dried over Na_2SO_4, filtered, and vacuum evaporated. The residue (4.48 g) was dissolved again in CH_2Cl_2 and treated with 2 N HCl. Both layers were then treated separately. The organic layer was washed with brine, dried, filtered, and evaporated to afford 2.20 g (52 %) of the uncharacterized compound *N*, *N*'-(4,5-dimethoxy-1,3-phenylene)bis(4-methoxybenzenesulfonamide).
The aqueous layer was then neutralized with 5 % $NaHCO_3$ and extracted with CH_2Cl_2. The resulting organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated under vacuum to afford 1.18 g (3.49 mmol, 21 %) of the desired compound **4**, which was purified by flash column chromatography (hexane/EtOAc 1:1) (916 mg, 16 %). M.p.: 132–133 °C (CH_2Cl_2/Hexane). ^{1}H NMR (400 MHz, CD_3OD): δ 3.64 (3H, *s*), 3.65 (3H, *s*), 3.74 (3H, *s*), 6.08 (1H, *d*, *J* = 2.4), 6.21 (1H, *d*, *J* = 2.4), 6.90 (2H, *d*, *J* = 8.8), 7.67 (2H, *d*, *J* = 8.8). ^{13}C NMR (100 MHz, CD_3OD): δ 54.7 (CH_3), 54.7 (CH_3), 58.9 (CH_3), 95.8 (CH), 101.8 (CH), 113.7 (2CH), 129.0 (2CH), 130.9 (C), 132.9 (C), 133.8 (C), 141.1 (C), 152.9 (C), 163.0 (C). HRMS ($C_{15}H_{18}N_2O_5S$ + H^+): calcd 339.1009 (M + H^+), found

339.1013.

***N*-(3-amino-4,5-dimethoxyphenyl)-4-methoxy-*N*-methylbenzenesulfonamide (5).** To 110 mg (0.32 mmol) of **4** and 45 mg (0.65 mmol) of KOH in CH_3CN (50 mL), 40.7 μL (0.65 mmol) of CH_3I were added and it was stirred for 24 h. The CH_3CN solution was concentrated under reduced pressure and the residue dissolved in EtOAc and washed with brine. The organics were dried over Na_2SO_4, filtered, and evaporated. The crude material (91 mg) was purified by column chromatography using hexane/EtOAc 1:1 as eluant to give 60 mg (0.17 mmol, 52 %) of **5.** ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.06 (3H, *s*), 3.70 (3H, *s*), 3.78 (3H, *s*), 3.84 (3H, *s*), 6.05 (1H, *d*, $J = 2.4$), 6.07 (1H, *d*, $J = 2.4$), 6.91 (2H, *d*, $J = 8.8$), 7.54 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 38.4 (CH_3), 55.6 (CH_3), 55.7 (CH_3), 59.9 (CH_3), 101.7 (CH), 107.0 (CH), 113.7 (2CH), 128.3 (C), 130.1 (2CH), 134.8 (C), 137.7 (C), 140.3 (C), 152.6 (C), 162.9 (C). HRMS ($C_{16}H_{20}N_2O_5S + H^+$): calcd 353.1166 ($M + H^+$), found 353.1171.

***N*-(2,3-dimethoxy-5-((4-methoxyphenyl)sulfonamido)phenyl) formamide (6).** Compound **4** (197 mg, 0.58 mmol) was stirred in a mixture of CH_2Cl_2 (40 mL), pyridine (1 mL), and formic acid (3 mL) at room temperature for 48 h. Then, the reaction mixture was poured onto ice and treated with 2 N HCl and 5 % $NaHCO_3$, washed to neutrality with brine, dried over anhydrous Na_2SO_4, filtered, and the solvent evaporated to produce 142 mg (0.39 mmol, 67 %) of crude reaction product **6**, which was purified by crystallization in methanol (104 mg, 0.28 mmol, 48 %). M.p.: 213–217 °C (MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 3.76 (3H, *s*), 3.77 (3H, *s*), 3.82 (3H, *s*), 6.61 (1H, *d*, $J = 2.8$), 6.98 (2H, *d*, $J = 9.2$), 7.66 (1H, *d*, $J = 2.8$), 7.72 (2H, *d*, $J = 9.2$), 8.25 (1H, *s*). ^{13}C NMR (100 MHz, DMSO-D6): δ 55.7 (CH_3), 55.7 (CH_3), 60.5 (CH_3), 100.3 (CH), 104.5 (CH), 114.4 (2CH), 129.2 (2CH), 131.2 (C), 131.7 (C), 133.9 (C), 134.1 (C), 152.1 (C), 160.3 (CH), 162.5 (C). HRMS ($C_{16}H_{18}N_2O_6S + H^+$): calcd 367.0958 ($M + H^+$), found 367.0963.

***N*-(3,4-dimethoxy-5-(methylamino)phenyl)-4-methoxybenzenesulfonamide (7).** To a solution of **6** (110 mg, 0.30 mmol) and $NaBH_4$ (17 mg, 0.45 mmol) in dry THF (15 mL) at 0 °C, trichloroacetic acid (74 mg, 0.45 mmol) diluted in dry THF (5 mL) was added dropwise under nitrogen atmosphere. The reaction mixture was stirred at 0 °C to room temperature for 48 h, concentrated and re-dissolved in EtOAc, washed with brine, dried over anhydrous Na_2SO_4, filtered and the solvent evaporated under vacuum to afford the compound **7** (99 mg, 0.28 mmol, 93 %). The crude reaction product was purified by crystallization (22 mg, 0.06 mmol, 21 %). M.p.: 156–161 °C (CH_2Cl_2/Hexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.71 (3H, *s*), 3.71 (3H, *s*), 3.73 (3H, *s*), 3.83 (3H, *s*), 5.89 (1H, *d*, $J = 2.4$), 6.06 (1H, *d*, $J = 2.4$), 6.26 (1H, *bs*), 6.89 (2H, *d*, $J = 8.8$), 7.71 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 31.4 (CH_3), 56.7 (CH_3), 56.9 (CH_3), 61.1 (CH_3), 96.5 (CH), 98.7 (CH), 115.2 (2CH), 130.7 (2CH), 131.8 (C), 133.7 (C), 134.5 (C), 144.7 (C), 153.4 (C), 164.1 (C). HRMS ($C_{16}H_{20}N_2O_5S + H^+$): calcd 353.1166 ($M + H^+$), found 353.1161.

***N*-(3,4-dimethoxy-5-(methylamino)phenyl)-4-methoxy-*N*-methylbenzenesulfonamide (8).** 58 mg (0.16 mmol) of **7** were dissolved in CH_3CN (25 mL) with 19 mg (0.33 mmol) of KOH. After 30 min stirring at room temperature, CH_3I (20.6 μL, 0.33 mmol) was added to the solution and the reaction mixture was stirred for 24 h. The solvent was removed under reduced pressure. The residue was dissolved in EtOAc and washed with brine, dried (Na_2SO_4), and concentrated in vacuo to afford 57 mg (0.15 mmol, 94 %) of **8**. The crude product was purified by crystallization in CH_2Cl_2/Hexane (11 mg, 0.03 mmol, 18 %). M.p.: 138–146 °C (CH_2Cl_2/Hexane). IR (KBr): 3411, 1602, 1497, 1175, 825 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.69 (3H, *d*, $J = 4.4$), 3.11 (3H, *s*), 3.72 (3H, *s*), 3.77 (3H, *s*), 3.86 (3H, *s*), 4.30 (1H, *bs*), 5.91 (1H, *d*, $J = 2.4$), 6.05 (1H, *d*, $J = 2.4$), 6.93 (2H, *d*, $J = 8.8$), 7.59 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.2 (CH_3), 38.6 (CH_3), 55.6 (CH_3), 55.7 (CH_3), 59.8 (CH_3), 100.6 (CH), 102.5 (CH), 113.7 (2CH), 128.5 (C), 130.2 (2CH), 134.3 (C), 138.1 (C), 143.1 (C), 151.8 (C), 162.9 (C). HRMS ($C_{17}H_{22}N_2O_5S + H^+$): calcd 367.1322 ($M + H^+$), found 367.1318.

***N*-(3-(dimethylamino)-4,5-dimethoxyphenyl)-4-methoxy-*N*-methylbenzenesulfonamide (9).** To a solution of **4** (100 mg, 0.29 mmol) and K_2CO_3 (408 mg, 2.96 mmol) in acetone (20 mL), $(CH_3)_2SO_4$ (212 μL, 2.21 mmol) was dropwise added and heated at reflux for 1 h. Then, the reaction mixture was filtered, poured onto ice, and extracted with CH_2Cl_2. The organic layers were dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness (50 mg). Compound **9** (8 mg, 0.02 mmol, 7 %) was isolated by preparative TLC (hexane/EtOAc 4:6). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.64 (6H, *s*), 3.05 (3H, *s*), 3.66 (3H, *s*), 3.72 (3H, *s*), 3.79 (3H, *s*), 6.97 (1H, *bs*), 6.23 (1H, *bs*), 6.86 (2H, *d*, $J = 8.4$), 7.48 (2H, *d*, $J = 8.4$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 38.6 (CH_3), 42.7 ($2CH_3$), 55.6 (CH_3), 56.0 (CH_3), 59.3 (CH_3), 104.9 (CH), 109.5 (CH), 113.7 (2CH), 128.4 (C), 130.2 (2CH), 137.1 (C), 140.2 (C), 146.2 (C), 153.1 (C), 162.9 (C). HRMS ($C_{18}H_{24}N_2O_5S + H^+$): calcd 381.1479 ($M + H^+$), found 381.1467.

***N*-(3,4-dimethoxyphenyl)-4-methoxybenzenesulfonamide (10).** To 2.49 g of 3,4-dimethoxyaniline (16.26 mmol) in CH_2Cl_2 (50 mL) and pyridine (4 mL), 3.61 g of 4-methoxybenzenesulfonyl chloride was slowly added (16.26 mmol) and stirred at room temperature for 12 h. The reaction was treated with 2 N HCl and 5 % $NaHCO_3$, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 5.29 g (16.3 mmol, 99 %) of **10**. The residue was crystallized in CH_2Cl_2/Hexane to afford the purified compound (4.04 g, 12.5 mmol, 75 %). M.p.: 101–102 °C (CH_2Cl_2/Hexane). IR (KBr): 3224, 1498, 801 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.75 (3H, *s*), 3.79 (3H, *s*), 3.80 (3H, *s*), 6.53 (1H, *dd*, $J = 8.8$ and 2.8), 6.66 (1H, *d*, $J = 8.8$), 6.70 (1H, *d*, $J = 2.8$), 6.86 (2H, *d*, $J = 8.8$), 7.66 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.5 (CH_3), 55.9 (CH_3), 55.9(CH_3), 107.7 (CH), 111.1 (CH), 114.0 (2CH), 115.4 (CH), 129.4 (2CH), 129.5 (C), 130.3 (C), 147.2 (C), 149.1 (C), 163.0 (C). HRMS ($C_{15}H_{17}NO_5S + H^+$): calcd 324.0900 ($M + H^+$), found 324.0906.

***N*-(4,5-dimethoxy-2-nitrophenyl)-4-methoxybenzenesulfonamide (11).** To a solution of **10** (588 mg, 1.82 mmol) in CH_3CN (50 mL) *tert*butyl nitrite (120 μL, 0.91 mmol) was added and stirred at 45 °C. After 1 h, additional 0.91 mmol *tert*butyl nitrite was added to the reaction mixture and it was stirred at 45 °C for 24 h. The mixture was poured into ice and basified with 5 % $NaHCO_3$ solution and extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4, and concentrated under vacuum to yield **11** (638 mg, 1.73 mmol, 95 %). The residue was purified by crystallization in CH_2Cl_2/Hexane to afford 527 mg (1.43 mmol, 78 %). M.p.: 152–154 °C (CH_2Cl_2/Hexane). IR (KBr): 3257, 1521, 1499, 804 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (3H, *s*), 3.87 (3H, *s*), 3.98 (3H, *s*), 6.90 (2H, *d*, $J = 9.2$), 7.35 (1H, *s*), 7.53 (1H, *s*), 7.23 (2H, *d*, $J = 9.2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.7 (CH_3), 56.3 (CH_3), 56.7 (CH_3), 103.0 (CH), 107.2 (CH), 114.4 (2CH), 129.4 (2CH), 129.7 (C), 129.9 (C), 130.3 (C), 145.2 (C), 155.4 (C), 163.6 (C). HRMS ($C_{15}H_{16}N_2O_7S + H^+$): calcd 369.0751 ($M + H^+$), found 369.0752.

***N*-(2-amino-4,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (12).** Pd (C) (10 mg) was added to a solution of **11** (620 mg, 1.68 mmol) in ethyl acetate (100 mL) and the reaction was stirred at room temperature under an H_2 atmosphere for 48 h. By filtration through Celite and solvent evaporation, 550 mg (1.63 mmol, 96 %) of crude reaction **12** was obtained. 374 mg (1.10 mmol, 65 %) of the purified compound was isolated by crystallization in CH_2Cl_2/Hexane. M.p.: 102–112 °C (CH_2Cl_2/Hexane). IR (KBr): 3265, 1458, 835 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.46 (3H, *s*), 3.74 (3H, *s*), 3.79 (3H, *s*), 5.72 (1H, *s*), 5.88 (1H, *s*), 6.23 (1H, *s*), 6.87 (2H, *d*, $J = 8.4$), 7.61 (2H, *d*, $J = 8.4$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.6 (CH_3), 55.8 (CH_3), 56.2 (CH_3), 101.0 (CH), 112.4 (C), 112.9 (CH), 114.1 (2CH), 129.8 (2CH), 130.4 (C), 139.0 (C), 141.5 (C), 149.6 (C), 163.1 (C). HRMS ($C_{15}H_{18}N_2O_5S + H^+$): calcd 339.1009 ($M + H^+$), found 339.1018.

***N*-(2-amino-4,5-dimethoxyphenyl)-4-methoxy-*N*-methylbenzenesulfonamide (13).** To a solution of **12** (167 mg, 0.49 mmol) in CH_3CN (25 mL) 68 mg of crushed KOH (0.98 mmol) and 62 μL of methyl iodide (0.98 mmol) were added and stirred at room temperature for 24 h. Then, the reaction mixture was concentrated, re-dissolved in CH_2Cl_2,

washed with brine, dried over anhydrous Na_2SO_4, filtered, and vacuum concentrated to produce 140 mg (0.39 mmol, 80 %) of **13**. The crude reaction product was purified by crystallization in MeOH (52 mg, 0.15 mmol, 30 %). M.p.: 140–141 °C (MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.11 (3H, *s*), 3.46 (3H, *s*), 3.82 (3H, *s*), 3.87 (3H, *s*), 5.80 (1H, *s*), 6.34 (1H, *s*), 6.97 (2H, *d*, $J = 8.8$), 7.65 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.2 (CH_3), 56.9 (CH_3), 57.0 (CH_3), 57.6 (CH_3), 101.9 (CH), 112.1 (CH), 115.2 (2CH), 119.4 (C), 129.8 (C), 131.6 (2CH), 141.5 (C), 142.2 (C), 151.1 (C), 164.4 (C). HRMS ($C_{16}H_{20}N_2O_5S$ + H^+): calcd 353.1166 (M + H^+), found 353.1179.

***N*-(4,5-dimethoxy-2-((4-methoxyphenyl)sulfonamido)phenyl) formamide (14).** A solution of **12** (100 mg, 0.29 mmol) and formic acid (16 μL, 0.35 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature for 72 h. The reaction mixture was washed with brine, dried, and concentrated under vacuum to produce 95 mg (0.26 mmol, 88 %) of crude reaction product **14**, which was purified by crystallization in CH_2Cl_2/Hexane (61 mg, 0.17 mmol, 56 %). M.p.: 130–132 °C (CH_2Cl_2/Hexane). IR (KBr): 3391, 1664, 1598, 1457 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.52 (3H, *s*), 3.84 (3H, *s*), 3.86 (3H, *s*), 6.03 (1H, *s*), 6.20 (1H, *s*), 6.92 (2H, *d*, $J = 8.8$), 7.59 (1H, *s*), 7.62 (2H, *d*, $J = 8.8$), 7.98 (1H, *bs*), 8.29 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.4 (CH_3), 55.5 (CH_3), 55.7 (CH_3), 106.0 (CH), 111.0 (CH), 113.9 (2CH), 117.7 (C), 127.7 (C), 129.3 (C), 129.6 (2CH), 145.4 (C), 148.2 (C), 159.5 (C), 163.1 (CH). HRMS ($C_{16}H_{18}N_2O_6S$ + H^+): calcd 367.0958 (M + H^+), found 367.0966.

***N*-(4,5-dimethoxy-2-(methylamino)phenyl)-4-methoxybenzenesulfonamide (15).** To a solution of **14** (85 mg, 0.23 mmol) and $NaBH_4$ (13 mg, 0.35 mmol) in dry THF (5 mL) at 0 °C, trichloroacetic acid (57 mg, 0.35 mmol) diluted in dry THF (5 mL) was added dropwise under nitrogen atmosphere. The reaction mixture was stirred at 0 °C to room temperature for 24 h, concentrated and re-dissolved in EtOAc, washed with brine, dried over anhydrous Na_2SO_4, filtered and the solvent vacuum evaporated. The residue (76 mg) was purified by preparative TLC (hexane/EtOAc 3:7) to afford compound **15** (28 mg, 0.08 mmol, 34 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.72 (3H, *s*), 3.39 (3H, *s*), 3.78 (3H, *s*), 3.79 (3H, *s*), 5.89 (1H, *s*); 6.17 (1H, *s*), 6.86 (2H, *d*, $J =$ 8.8), 7.60 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 31.1 (CH_3), 55.6 (CH_3), 55.7 (CH_3), 55.9 (CH_3), 96.5 (CH), 111.6 (CH), 113.9 (2CH), 129.6 (C), 129.8 (2CH), 130.4 (C), 131.2 (C), 142.5 (C), 142.6 (C), 163.1 (C). HRMS ($C_{16}H_{20}N_2O_5S$ + H^+): calcd 353.1166 (M + H^+), found 353.1175.

***N*-(2-(dimethylamino)-4,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (16).** Compound **12** (149 mg, 0.44 mmol) was dissolved in MeOH (30 mL) with paraformaldehyde (50 mg, 1.66 mmol) and acetic acid (one drop). After 1 h stirring at room temperature sodium cyanoborohydride (28 mg, 0.44 mmol) was added to the solution. The reaction mixture was allowed to react for 4 days. The crude reaction product was purified by flash chromatography in Hexane/EtOAc 6:4 to afford **16** (65 mg, 0.18 mmol, 40 %). M.p.: 165–169 °C (MeOH). IR (KBr): 3436, 1594, 1515, 835 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.23 (6H, *s*), 3.73 (6H, *s*), 3.81 (3H, *s*), 6.56 (1H, *s*), 6.78 (2H, *d*, $J = 8.8$), 7.62 (2H, *d*, $J = 8.8$), 7.83 (1H, *bs*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 45.5 ($2CH_3$), 55.5 ($2CH_3$), 56.1 (CH_3), 103.3 (CH), 105.1 (CH), 113.9 (2CH), 126.6 (C), 129.2 (2CH), 130.9 (C), 136.1 (C), 145.8 (C), 146.8 (C), 162.9 (C). HRMS ($C_{17}H_{22}N_2O_5S$ + H^+): calcd 367.1322 (M + H^+), found 367.1328.

***N*-(4,5-dimethoxy-2-((4-methoxyphenyl)sulfonamido)phenyl) acetamide (17a) and *N*-(2-acetamido-4,5-dimethoxyphenyl)-*N*-((4-methoxyphenyl)sulfonyl)acetamide (17b).** To a solution of **12** (200 mg, 0.59 mmol) in pyridine (1 mL), 555 μL of acetic anhydride (5.90 mmol) was added. The reaction mixture was stirred for 30 min. The mixture was poured onto ice and extracted with EtOAc. The organic layer was washed with 2 N HCl, 5 % $NaHCO_3$, and brine, dried (Na_2SO_4), filtered, and concentrated in vacuo to yield 198 mg of crude reaction residue. The residue was purified by preparative TLC (CH_2Cl_2/MeOH 95:5) to afford compounds **17a** (31 mg, 0.08 mmol, 14 %) and **17b** (44 mg, 0.10 mmol, 18 %). **17a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.07 (3H, *s*), 3.52 (3H, *s*), 3.79 (6H, *s*), 6.29 (1H, *s*), 6.85 (2H, *d*, $J = 8.8$), 7.34 (1H, *s*), 7.54 (2H, *d*, $J = 8.8$), 7.67 (1H, *bs*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.2 (CH_3), 55.7 (CH_3), 55.9 (CH_3), 56.0 (CH_3), 106.4 (CH), 111.5 (CH), 114.1 (2CH), 118.6 (C), 128.3 (C), 129.7 (2CH), 130.1 (C), 145.8 (C), 148.5 (C), 163.3 (C), 169.0 (C). HRMS ($C_{17}H_{20}N_2O_6S$ + H^+): calcd 381.1115 (M + H^+), found 381.1124. **17b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.86 (3H, *s*), 2.17 (3H, *s*), 3.78 (3H, *s*), 3.90 (3H, *s*), 3.94 (3H, *s*), 6.40 (1H, *s*), 7.02 (2H, *d*, $J = 8.8$), 7.46 (1H, *s*), 7.79 (1H, *s*), 7.99 (2H, *d*, $J =$ 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.4 (CH_3), 24.5 (CH_3), 55.8 (CH_3), 56.2 (CH_3), 56.3 (CH_3), 107.5 (CH), 111.5 (CH), 114.1 (2CH), 119.4 (C), 129.3 (C), 130.9 (C), 131.7 (2CH), 146.4 (C), 150.5 (C), 164.3 (C), 168.9 (C), 171.0 (C). HRMS ($C_{19}H_{22}N_2O_7S$ + H^+): calcd 423.1220 (M + H^+), found 423.1225.

***N*-(4,5-dimethoxy-2-((4-methoxy-*N*-methylphenyl)sulfonamido)phenyl)acetamide (18).** 90 mg of **13** (0.25 mmol) were dissolved in CH_2Cl_2 (45 mL) and pyridine (1 mL). 29 μL of acetic anhydride (0.30 mmol) were added to the solution and stirred at room temperature for 24 h. The reaction mixture was poured onto ice and treated with 2 N HCl and 5 % $NaHCO_3$. The organic layers were washed to neutrality with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue (90 mg) was purified by silica preparative chromatography (hexane/EtOAc 2:8) to afford **18** (56 mg, 0.14 mmol, 55 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.21 (3H, *s*), 3.10 (3H, *s*), 3.44 (3H, *s*), 3.85 (3H, *s*), 3.88 (3H, *s*), 5.75 (1H, *s*), 6.96 (2H, *d*, $J = 8.8$), 7.55 (2H, *d*, $J = 8.8$), 7.89 (1H, *s*), 8.21 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.8 (CH_3), 39.5 (CH_3), 55.6 (CH_3), 55.8 (CH_3), 56.0 (CH_3), 105.9 (CH), 108.9 (CH), 114.0 (2CH), 122.5 (C), 127.1 (C), 130.4 (C), 130.8 (2CH), 144.8 (C), 148.8 (C), 163.5 (C), 168.6 (C). HRMS ($C_{18}H_{22}N_2O_6S$ + H^+): calcd 395.1271 (M + H^+), found 395.1280.

4-((4,5-dimethoxy-2-((4-methoxyphenyl)sulfonamido)phenyl) amino)-4-oxobutanoic acid (19). A mixture of **12** (132 mg, 0.39 mmol), succinic anhydride (47 mg, 0.47 mmol), and pyridine drops in CH_2Cl_2 (50 mL) was stirred at room temperature. After 24 h, the precipitate formed was filtered to afford 100 mg (0.23 mmol, 59 %) of **19**. IR (KBr): 3365, 3273, 1707, 1689, 1597 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 2.53 (2H, *t*, $J = 6.4$), 2.63 (2H, *t*, $J = 6.4$), 3.67 (3H, *s*), 3.76 (3H, *s*), 3.84 (3H, *s*), 6.61 (1H, *s*), 6.99 (2H, *d*, $J = 9.2$), 7.01 (1H, *s*), 7.56 (2H, *d*, $J = 9.2$). ^{13}C NMR (100 MHz, CD_3OD): δ 28.4 (CH_2), 30.5 (CH_2), 54.7 (CH_3), 55.0 (CH_3), 55.1 (CH_3), 107.1 (CH), 111.2 (CH), 113.7 (2CH), 121.0 (C), 126.7 (C), 129.1 (2CH), 130.7 (C), 146.5 (C), 147.9 (C), 163.3 (C), 171.5 (C), 174.8 (C). HRMS ($C_{19}H_{22}N_2O_8S$ + H^+): calcd 439.1170 (M + H^+), found 439.1184.

***Tert*-butyl (2-((4,5-dimethoxy-2-((4-methoxyphenyl)sulfonamido)phenyl)amino)-2-oxoethyl)carbamate (20).** A solution of (*tert*-butoxycarbonyl)glycine (135 mg, 0.77 mmol), EDCI (138 mg, 0.89 mmol) and DMAP (36 mg, 0.29 mmol) in CH_2Cl_2 (20 mL) was stirred for 10 min. Then, to this solution was added the compound **12** (200 mg, 0.59 mmol) and the reaction mixture was stirred at room temperature for 24 h. The solution was washed with 2 N HCl, 5 % $NaHCO_3$, and brine. After drying (Na_2SO_4) and removal of the solvent (207 mg), compound **20** was isolated by flash column chromatography using eluent CH_2Cl_2 /EtOAc 8:2 (88 mg, 0.18 mmol, 30 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.51 (9H, *s*), 3.59 (3H, *s*), 3.85 (6H, *s*), 3.89 (2H, *d*, $J = 6.4$), 6.21 (1H, *s*), 6.49 (1H, *s*), 6.92 (2H, *d*, $J = 8.8$), 7.42 (1H, *s*), 7.60 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.7 ($3CH_3$), 45.1 (CH_2), 56.0 (CH_3), 56.2 (CH_3), 56.3 (CH_3), 80.9 (C), 106.9 (CH), 111.5 (CH), 114.4 (2CH), 119.6 (C), 127.8 (C), 130.1 (2CH), 130.5 (C), 146.3 (C), 148.5 (C), 156.7 (C), 163.6 (C), 168.9 (C). HRMS ($C_{22}H_{29}N_3O_8S$ + H^+): calcd 496.1748 (M + H^+), found 496.1746.

5,6-Dimethoxy-1-((4-methoxyphenyl)sulfonyl)-1*H*-benzo [*d*] [1–3]triazole (21). To a solution of **12** (57 mg, 0.17 mmol) in CH_3CN (10 mL) and H_2O (100 μL) at 0 °C, *tert*butyl nitrite (22.5 μL, 0.17 mmol) was added and the reaction stirred. After 1 h, acetic acid (10 μL) was added to the reaction mixture and stirred for 24 h to room temperature. Then, the mixture was concentrated, re-dissolved in EtOAc, and treated with a 5 % $NaHCO_3$ solution. The organic layers were washed with brine

to neutrality, dried over Na_2SO_4, and vacuum concentrated. The residue (58 mg) was chromatographed on silica TLC preparative (CH_2Cl_2/EtOAc 9:1) to afford the purified compound **21** (17 mg, 0.05 mmol, 29 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (3H, *s*), 3.94 (3H, *s*), 4.05 (3H, *s*), 6.96 (2H, *d*, $J = 8.8$), 7.34 (1H, *s*), 7.44 (1H, *s*), 8.02 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.8 (CH_3), 56.3 (CH_3), 56.6 (CH_3), 92.5 (CH), 99.3 (CH), 114.8 (2CH), 127.2 (C), 128.2 (C), 130.3 (2CH), 139.7 (C), 149.4 (C), 153.1 (C), 164.9 (C). HRMS ($C_{15}H_{15}N_3O_5S + H^+$): calcd 350.0805 ($M + H^+$), found 350.0796.

5,6-Dimethoxy-1-((4-methoxyphenyl)sulfonyl)-1*H*-benzo[*d*] imidazole (22). A mixture of **12** (100 mg, 0.29 mmol) and triethyl orthoformate (60 μL, 0.35 mmol) in CH_3CN (10 mL) was heated at reflux for 12 h. The CH_3CN was removed under reduced pressure and the residue was washed with brine, extracted with EtOAc, and dried over Na_2SO_4. After concentration, crude reaction product **22** (100 mg, 0.29 mmol, 97 %) was purified by crystallization (81 mg, 0.23 mmol, 79 %). M.p.: 175–177 °C (CH_2Cl_2/Hexane). IR (KBr): 1633, 1617, 1592 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (3H, *s*), 3.90 (3H, *s*), 3.97 (3H, *s*), 6.94 (2H, *d*, $J = 9.2$), 7.20 (1H, *s*), 7.33 (1H, *s*), 6.88 (2H, *d*, $J = 9.2$), 8.21 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.8 (CH_3), 56.2 (CH_3), 56.5 (CH_3), 95.2 (CH), 102.5 (CH), 114.8 (2CH), 124.5 (C), 128.9 (C), 129.3 (2CH), 137.5 (C), 139.8 (CH), 148.1 (C), 148.7 (C), 164.4 (C). HRMS ($C_{16}H_{16}N_2O_5S + H^+$): calcd 349.0853 ($M + H^+$), found 349.0841.

1,4-Dimethoxy-2-nitrobenzene (23). To a solution of 1,4-dimethoxybenzene (2.35 g, 17 mmol) in acetic acid (30 mL) at 0 °C, nitric acid (1.13 mL, 17 mmol) in acetic acid (20 mL) was added dropwise under a nitrogen atmosphere. The reaction mixture was stirred at 0 °C for 4 h and then poured onto ice with 5 % $NaHCO_3$ and extracted with EtOAc. The organic layers were washed to neutrality with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in a vacuum to obtain 2.92 g (15.9 mmol, 94 %) of **23**. M.p.: 71.8–72.5 °C (CH_2Cl_2/Hexane). IR (KBr): 1528, 874, 763 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.82 (3H, *s*), 3.92 (3H, *s*), 7.03 (1H, *d*, $J = 9.6$), 7.12 (1H, *dd*, $J = 9.6$ and 3.2), 7.4 (1H, *d*, $J = 3.2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.9 (CH_3), 56.9 (CH_3), 109.9 (CH), 115.0 (CH), 120.8 (CH), 139.3 (C), 147.3 (C), 152.7 (C). GC–MS ($C_8H_9NO_4^+$): 183 (M^+).

2,5-Dimethoxyaniline (24). 1,4-dimethoxy-2-nitrobenzene (**23**, 2.92 g, 15.95 mmol) was dissolved in EtOAc (100 mL) and was palladium-catalyzed (Pd (C) 10 mg) reduced under H_2 atmosphere for 48 h. The reaction mixture was filtered through Celite and the solvent evaporated in a vacuum to isolate 2.42 g (15.8 mmol, 99 %) of **24**. The crude reaction product was obtained and used without further purification. IR (KBr): 3459, 1519, 839 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.72 (3H, *s*), 3.79 (3H, *s*), 6.24 (1H, *dd*, $J = 9.2$ and 3.2), 6.33 (1H, *d*, $J = 3.2$), 6.69 (1H, *d*, $J = 9.2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.5 (CH_3), 55.1 (CH_3), 100.9 (CH), 101.1 (CH), 110.3 (CH), 136.2 (C), 140.9 (C), 153.3 (C). GC–MS ($C_8H_{11}NO_2^+$): 153 (M^+).

***N*-(2,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (25).** To a solution of **24** (2.42 g, 15.84 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL), 4-methoxybenzenesulfonyl chloride (3.27 g, 15.84 mmol) was slowly added. The mixture was stirred at room temperature for 4 h. Then the reaction was treated with 2 N HCl and 5 % $NaHCO_3$, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 4.9 g (15.2 mmol, 95 %) of **25**. It was purified by crystallization in CH_2Cl_2/Hexane (4.29 g, 13.3 mmol, 84 %). M.p.: 114–115 °C (CH_2Cl_2/Hexane). IR (KBr): 3313, 1578, 830 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.62 (3H, *s*), 3.74 (3H, *s*), 3.81 (3H, *s*), 6.53 (1H, *dd*, $J = 9.2$ and 3.2), 6.65 (1H, *d*, $J = 9.2$), 6.86 (2H, *d*, $J = 9.2$), 7.01 (1H, *bs*), 7.14 (1H, *d*, $J = 3.2$), 7.72 (2H, *d*, $J = 9.2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.5 (CH_3), 55.7 (CH_3), 56.2 (CH_3), 196.8 (CH), 109.5 (CH), 111.4 (CH), 113.9 (2CH), 126.8 (C), 129.4 (2CH), 130.7 (C), 143.4 (C), 153.8 (C), 163.0 (C). HRMS ($C_{15}H_{17}NO_5S + H^+$): calcd 324.0900 ($M + H^+$), found 324.0900.

***N*-(2,5-dimethoxy-4-nitrophenyl)-4-methoxybenzenesulfonamide (26).** To a stirred solution at 0 °C of **25** (2.07 g, 6.42 mmol) in acetic acid (30 mL), nitric acid (0.44 mL, 6.42 mmol) in acetic acid (20 mL) was slowly added under a nitrogen atmosphere. After 4 h at 0 °C, the reaction mixture was poured onto ice and basified with a 5 % $NaHCO_3$ solution. Then it was extracted with EtOAc. The organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in a vacuum to obtain 2.14 g (5.81 mmol, 90 %) of **26**. By crystallization in CH_2Cl_2/Hexane, 1.53 g (4.16 mmol, 65 %) of the purified product was isolated. M.p.: 161–163 °C (CH_2Cl_2/Hexane). IR (KBr): 3277, 1522, 1450, 822 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.76 (3H, *s*), 3.79 (3H, *s*), 3.88 (3H, *s*), 6.9 (2H, *d*, $J = 9.2$), 7.39 (1H, *s*), 7.56 (1H, *s*), 7.77 (2H, *d*, $J = 9.2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.7 (CH_3), 56.6 (CH_3), 57.0 (CH_3), 103.2 (CH), 108.3 (CH), 114.4 (2CH), 129.4 (2CH), 129.9 (C), 132.8 (C), 133.1 (C), 141.1 (C), 149.2 (C), 163.6 (C). HRMS ($C_{15}H_{16}N_2O_7S + H^+$): calcd 369.0751 ($M + H^+$), found 369.0753.

***N*-(4-amino-2,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (27).** The nitro sulfonamide **26** (1.44 g, 3.91 mmol) in EtOAc (100 mL) and Pd (C) (10 mg) was stirred at room temperature under an H_2 atmosphere for 48 h. By filtration through Celite and solvent evaporation, hydrogenated sulfonamide **27** (1.28 g, 3.79 mmol, 97 %) was obtained. 1,11 g (3.28 mmol, 84 %) of **27** were isolated by crystallization in CH_2Cl_2/Hexane. M.p.: 164–166 °C (CH_2Cl_2/Hexane). IR (KBr): 3430, 3291, 1451, 834 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.38 (3H, *s*), 3.79 (3H, *s*), 3.82 (3H, *s*), 6.15 (1H, *s*), 6.54 (1H, *bs*), 6.82 (2H, *d*, $J = 9.2$), 7.04 (1H, *s*), 7.58 (2H, *d*, $J = 9.2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.5 (CH_3), 55.9 (CH_3), 56.2 (CH_3), 99.2 (CH), 108.4 (CH), 113.5 (2CH), 115.3 (C), 129.4 (2CH), 130.7 (C), 134.6 (C), 141.0 (C), 145.8 (C), 162.7 (C). HRMS ($C_{15}H_{18}N_2O_5S + H^+$): calcd 339.1009 ($M + H^+$), found 339.1012.

***N*-(2,5-dimethoxy-4-((4-methoxyphenyl)sulfonamido)phenyl) formamide (28).** Sulfonamide **27** (180 mg, 0.53 mmol) was dissolved in CH_2Cl_2 (50 mL) and pyridine (1 mL). 48 μL (1.06 mmol) of formic acid was added and stirred at room temperature for 48 h. The reaction mixture was washed with 2 N HCl, 5 % $NaHCO_3$, and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo to afford 174 mg (0.47 mmol, 89 %) of **28**. The crude reaction product was purified by preparative TLC in hexane/EtOAc 2:8 (146 mg, 0.40 mmol, 75 %). IR (KBr): 3325, 3253, 1664, 1498, 841 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.57 (3H, *s*), 3.81 (3H, *s*), 3.87 (3H, *s*), 6.85 (2H, *d*, $J = 8.8$), 7.19 (1H, *s*), 7.63 (2H, *d*, $J = 8.8$) 7.97 (1H, *s*), 8.39 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.9 (CH_3), 56.6 (CH_3), 56.8 (CH_3), 104.7 (CH), 106.2 (CH), 114.3 (2CH), 121.4 (C), 124.6 (C), 129.6 (2CH), 130.9 (C), 142.2 (C), 144.4 (C), 159.3 (CH), 163.4 (C). HRMS ($C_{16}H_{18}N_2O_6S + Na^+$): calcd 389.0778 ($M + Na^+$), found 389.0786.

***N*-(2,5-dimethoxy-4-(methylamino)phenyl)-4-methoxybenzenesulfonamide (29).** To a solution of **28** (185 mg, 0.50 mmol) and $NaBH_4$ (29 mg, 0.76 mmol) in dry THF (10 mL) at 0 °C, trichloroacetic acid (124 mg, 0.76 mmol) diluted in dry THF (2 mL) was added dropwise under nitrogen atmosphere. The reaction mixture was stirred at 0 °C to room temperature for 24 h, concentrated and re-dissolved in EtOAc, washed with brine, dried over anhydrous Na_2SO_4, filtered and the solvent evaporated in a vacuum to afford 175 mg of crude reaction. Compound **29** (142 mg, 0.40 mmol, 80 %) was isolated by flash column chromatography (hexane/EtOAc 6:4). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.80 (3H, *s*), 3.40 (3H, *s*), 3.81 (3H, *s*), 3.82 (3H, *s*), 5.97 (1H, *s*), 6.49 (1H, *bs*), 6.82 (2H, *d*, $J = 8.8$), 7.00 (1H, *s*), 7.58 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.3 (CH_3), 55.5 (CH_3), 56.0 (CH_3), 56.1 (CH_3), 94.0 (CH), 107.6 (CH), 112.9 (C), 113.5 (2CH), 129.4 (2CH), 130.8 (C), 138.3 (C), 140.6 (C), 146.5 (C), 162.7 (C). HRMS ($C_{16}H_{20}N_2O_5S + H^+$): calcd 353.1166 ($M + H^+$), found 353.1164.

***N*-(2,5-dimethoxy-4-(methylamino)phenyl)-4-methoxy-*N*-methylbenzenesulfonamide (30).** To a solution of **27** (149 mg, 0.44 mmol) in acetone (50 mL), K_2CO_3 (609 mg, 4.40 mmol) and $(CH_3)_2SO_4$ (316 μL, 3.30 mmol) were added, heated at reflux, and stirred overnight. Then, the reaction mixture was filtered, poured onto ice, and extracted with CH_2Cl_2. The organic layers were dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness to afford 70 mg of crude reaction. By

preparative TLC (toluene/EtOAc 4:6) compound **30** (25 mg, 0.07 mmol, 15 %) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.84 (3H, *s*), 3.18 (3H, *s*), 3.38 (3H, *s*), 3.76 (3H, *s*), 3.86 (3H, *s*), 6.01 (1H, *s*), 6.67 (1H, *s*), 6.91 (2H, *d, J* = 9.2), 7.64 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.1 (CH_3), 38.2 (CH_3), 55.3 (CH_3), 55.5 (CH_3), 55.9 (CH_3), 93.8 (CH), 112.9 (CH), 113.4 (2CH), 115.9 (C), 129.8 (2CH), 131.6 (C), 139.9 (C), 140.2 (C), 151.5 (C), 162.4 (C). HRMS ($C_{17}H_{22}N_2O_5S$ + H^+): calcd 367.1322 (M + H^+), found 367.1321.

***N*-(2,5-dimethoxy-4-((4-methoxyphenyl)sulfonamido)phenyl) acetamide (31).** A mixture of **27** (97 mg, 0.28 mmol), acetic anhydride (32 μL, 0.34 mmol) and pyridine (2 mL) in CH_2Cl_2 (50 mL) was stirred at room temperature for 24 h. Then, it was poured onto ice and extracted with CH_2Cl_2. Organic layers were washed with 2 N HCl, 5 % $NaHCO_3$, and brine, dried (Na_2SO_4), filtered, and evaporated to dryness to give 98 mg (0.26 mmol, 90 %) of **31**. The crude reaction product was purified by crystallization in CH_2Cl_2/Hexane (54 mg, 0.14 mmol, 49 %). M.p.: 178–184 °C (CH_2Cl_2/Hexane). IR (KBr): 3220, 1668, 1598, 1499, 829 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.15 (3H, *s*), 3.52 (3H, *s*), 3.78 (3H, *s*), 3.83 (3H, *s*), 6.81 (2H, *d, J* = 9.2), 7.14 (1H, *s*), 7.60 (2H, *d, J* = 9.2), 7.72 (1H, *bs*), 7.97 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.9 (CH_3), 55.6 (CH_3), 56.2 (CH_3), 56.4 (CH_3), 103.6 (CH), 105.7 (CH), 113.8 (2CH), 120.4 (C), 125.3 (C), 129.3 (2CH), 130.5 (C), 141.5 (C), 144.2 (C), 162.9 (C), 168.2 (C). HRMS ($C_{17}H_{20}N_2O_6S$ + H^+): calcd 381.1115 (M + H^+), found 381.1126.

***N*-(2,5-dimethoxy-4-((4-methoxy-*N*-methylphenyl)sulfonamido)phenyl)-*N*-methylacetamide (32).** 80 mg (0.21 mmol) of **31** were dissolved in CH_3CN (40 mL) and 23 mg (0.42 mmol) of KOH were added. After 30 min stirring at room temperature, CH_3I (26 μL, 0.42 mmol) was added to the solution. The reaction mixture was stirred for 24 h, concentrated under reduced pressure, dissolved in EtOAc, washed with brine, dried (Na_2SO_4), filtered, and evaporated to dryness. 72 mg (0.17 mmol, 87 %) of compound **32** were yielded and purified by crystallization in CH_2Cl_2/Hexane (44 mg, 0.11 mmol, 53 %). M.p.: 170–171 °C (CH_2Cl_2/Hexane). IR (KBr): 1676, 1654, 1597, 1514, 834 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.82 (3H, *s*), 3.15 (3H, *s*), 3.20 (3H, *s*), 3.40 (3H, *s*), 3.78 (3H, *s*), 3.87 (3H, *s*), 6.60 (1H, *s*), 6.93 (2H, *d, J* = 9.2), 6.97 (1H, *s*), 7.65 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.7 (CH_3), 35.9 (CH_3), 37.7 (CH_3), 55.5 (CH_3), 55.6 (CH_3), 56.0 (CH_3), 112.2 (CH), 113.6 (2CH), 116.0 (CH), 129.2 (C), 129.7 (2CH), 131.0 (C), 132.9 (C), 148.6 (C), 150.3 (C), 162.8 (C), 171.1 (C). HRMS ($C_{19}H_{24}N_2O_6S$ + H^+): calcd 409.1428 (M + H^+), found 409.1413.

2-Chloro-*N*-(2,5-dimethoxy-4-((4-methoxyphenyl)sulfonamido) phenyl)acetamide (33). To a solution of amine **27** (160 mg, 0.47 mmol) in CH_2Cl_2 (25 mL), 2-chloroacetyl chloride (46.4 μL, 0.57 mmol) was added dropwise under nitrogen atmosphere. After 12 h at room temperature, the reaction was washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated in a vacuum to give 171 mg (0.41 mmol, 87 %) of **33.** The crude reaction product was purified by crystallization in methanol (55 mg, 0.13 mmol, 28 %). M.p.: 175–177 °C (MeOH). IR (KBr): 3372, 3265, 1677, 1598, 829 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 3.47 (3H, *s*), 3.81 (3H, *s*), 3.86 (3H, *s*), 4.25 (2H, *s*), 6.94 (2H, *d, J* = 8.8), 7.14 (1H, *s*), 7.62 (2H, *d, J* = 8.8), 7.74 (1H, *s*). ^{13}C NMR (100 MHz, Acetone-D6): δ 43.2 (CH_2), 55.1 (CH_3), 55.8 (CH_3), 56.0 (CH_3), 104.1 (CH), 106.9 (CH), 113.8 (2CH), 124.8 (C), 129.3 (2CH), 131.4 (C), 138.1 (C), 142.4 (C), 144.9 (C), 163.0 (C), 164.1 (C). HRMS ($C_{17}H_{19}ClN_2O_6S$ + H^+): calcd 415.0725 (M + H^+), found 415.0700.

3-Chloro-*N*-(2,5-dimethoxy-4-((4-methoxyphenyl)sulfonamido) phenyl)propanamide (34). To a stirred solution at room temperature of **27** (143 mg, 0.42 mmol) in CH_2Cl_2 (30 mL) 3-chloropropanoyl chloride (49.4 μL, 0.51 mmol) was slowly added under nitrogen atmosphere. After 12 h, the reaction mixture was crystalized in CH_2Cl_2 to obtain 88 mg (0.20 mmol, 48 %) of **34**. M.p.: 193–197 °C (CH_2Cl_2). ^{1}H NMR (400 MHz, CD_3OD): δ 2.89 (2H, *t, J* = 6.4), 3.47 (3H, *s*), 3.81 (3H, *s*), 3.83 (3H, *s*), 3.83 (2H, *t, J* = 6.4), 6.95 (2H, *d, J* = 9.2), 7.12 (1H, *s*), 7.63 (2H, *d, J* = 9.2), 7.68 (1H, *s*). ^{13}C NMR (100 MHz, Acetone-D6): δ 39.6 (CH_2), 40.1 (CH_2), 55.1 (CH_3), 55.7 (CH_3), 55.9 (CH_3), 104.4 (CH), 107.1 (CH), 113.7 (2CH), 120.9 (C), 125.7 (C), 129.3 (2CH), 131.7 (C), 142.2 (C), 144.9 (C), 162.9 (C), 167.8 (C). HRMS ($C_{18}H_{21}ClN_2O_6S$ + H^+): calcd 429.0882 (M + H^+), found 429.0879.

***N*-(4-(3-ethylureido)-2,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (35).** To 98 mg (0.29 mmol) of **27** in CH_2Cl_2 (7 mL), 34 μL (0.43 mmol) of ethyl isocyanate were added. After 6 h stirring at room temperature, the precipitate formed was filtered to afford 87 mg (0.21 mmol, 73 %) of **35**. The obtained solid was recrystallized from CH_2Cl_2 to give pure product **35** (70 mg, 0.17 mmol, 59 %). M.p.: 208–211 °C (CH_2Cl_2). IR (KBr): 3354, 3231, 1651, 1498, 829 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.18 (3H, *t, J* = 7.2), 3.28 (2H, *m*), 3.51 (3H, *s*), 3.81 (3H, *s*), 3.83 (3H, *s*), 4.50 (1H, *bs*), 6.71 (1H, *bs*), 6.74 (1H, *bs*), 6.83 (2H, *d, J* = 9.2), 7.13 (1H, *s*), 7.60 (2H, *d, J* = 9.2), 7.79 (1H, *s*). ^{13}C NMR (100 MHz, Acetone-D6): δ 14.8 (CH_3), 34.2 (CH_3), 55.1 (CH_3), 55.5 (CH_3), 55.9 (CH_3), 102.5 (CH), 107.8 (CH), 113.6 (2CH), 118.1 (C), 128.4 (C), 129.3 (2CH), 131.9 (C), 141.0 (C), 145.7 (C), 154.9 (C), 162.9 (C). HRMS ($C_{18}H_{23}N_3O_6S$ + H^+): calcd 410.1380 (M + H^+), found 410.1374.

***N*-(4-(3-ethyl-1-methylureido)-2,5-dimethoxyphenyl)-4-methoxy-*N*-methylbenzenesulfonamide (36).** To a stirred solution of **35** (60 mg, 0.15 mmol) in CH_3CN (25 mL), 17 mg (0.30 mmol) of KOH were added. After 30 min at room temperature, CH_3I (19 μL, 0.30 mmol) was added. The solution was stirred for 24 h; then the mixture was evaporated to dryness. The residue was dissolved with EtOAc and washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness to give 64 mg (0.15 mmol, 98 %) of **36**. The residue was purified by preparative TLC (CH_2Cl_2/MeOH 98:2) (57 mg, 0.13 mmol, 87 %) and recrystallized from MeOH (35 mg, 0.08 mmol, 53 %). M.p.: 120–123 °C (MeOH). IR (KBr): 3436, 1648, 1597, 807 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 1.04 (3H, *t, J* = 6.8), 3.09 (3H, *s*), 3.13 (2H, *q, J* = 6.8), 3.16 (3H, *s*), 3.44 (3H, *s*), 3.75 (3H, *s*), 3.88 (3H, *s*), 6.83 (1H, *s*), 6.93 (1H, *s*), 7.07 (2H, *d, J* = 9.2), 7.66 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, CD_3OD): δ 14.6 (CH_3), 35.0 (CH_3), 35.4 (CH_3), 36.7 (CH_2), 54.8 ($2CH_3$), 55.2 (CH_3), 113.4 (CH), 113.5 (2CH), 115.8 (CH), 128.8 (C), 129.5 (2CH), 130.7 (C), 131.8 (C), 149.1 (C), 150.9 (C), 158.7 (C), 163.2 (C). HRMS ($C_{20}H_{27}N_3O_6S$ + H^+): calcd 438.1693 (M + H^+), found 438.1689.

3,4-Dimethoxy-5-nitrobenzaldehyde (37). To a solution of 4-hydroxy-3-methoxy-5-nitrobenzaldehyde (5 g, 25.36 mmol) and K_2CO_3 (12.3 g, 89 mmol) in acetone (40 mL), $(CH_3)_2SO_4$ (12 mL, 127 mmol) was dropwise added. The reaction mixture was stirred at room temperature for 48 h and heated at reflux for 8 h. Then, it was filtered through Celite and concentrated in a vacuum. The residue (5.8 g) was purified by flash column chromatography (CH_2Cl_2/hexane 8:2) to afford compound **37** (4.36 g, 20.65 mmol, 81 %). M.p.: 91–92 °C (2-propanol). IR (KBr): 2955, 1699, 1539, 1363, 1287, 1139, 980 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.00 (3H, *s*), 4.08 (3H, *s*), 7.62 (1H, *d, J* = 2), 7.84 (1H, *d, J* = 2), 9.92 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.6 (CH_3), 62.2 (CH_3), 113.5 (CH), 119.3 (CH), 131.3 (C), 147.6 (C), 154.6 (C), 155.2 (C), 189.0 (CH).

(3,4-Dimethoxy-5-nitrophenyl)(4-methoxyphenyl)methanol (38). 4.25 g (22.73 mmol) of 1-bromo-4-methoxybenzene were dissolved in dry THF (30 mL) and cooled to −78 °C, then 9.5 mL (15.2 mmol) of *n*-BuLi 1.6 M in hexane were added. After 1 h stirring at −78 °C, the aldehyde **37** (1.6 g, 7.58 mmol) was added to the solution. The reaction mixture was stirred for 24 h at room temperature, treated with NH_4Cl, extracted with EtOAc, and evaporated to dryness. 2.30 g (7.52 mmol, 96 %) of crude reaction product **38** was obtained and used without further purification. IR (KBr): 3407, 1595, 1512, 1455, 1352, 1244, 1173, 832 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.70 (6H, *s*), 3.76 (3H, *s*), 5.74 (1H, *s*), 6.90 (2H, *d, J* = 9), 7.15 (1H, *s*), 7.30 (2H, *d, J* = 9), 7.77 (1H, *s*).

(3,4-Dimethoxy-5-nitrophenyl)(4-methoxyphenyl)methanone (39). Compound **38** (2.30 g, 7.52 mmol) was dissolved in CH_2Cl_2 and cooled to 0 °C. PDC (6.2 g, 16.44 mmol) was added and stirred at room temperature for 4 h. The reaction mixture was filtered through Celite and evaporated to dryness. The crude residue (2.4 g) was purified by

flash column chromatography (hexane/EtOAc 7:3) to yield 895 mg (2.82 mmol, 37 %) of pure product **39**. M.p.: 117–118 °C (CH_2Cl_2/Hexane). IR (KBr): 1653, 1605, 1533, 1362, 1298, 1172, 844 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.90 (3H, *s*), 3.98 (3H, *s*), 4.07 (3H, *s*), 6.97 (2H, *d*, $J = 9$), 7.59 (1H, *d*, $J = 2$), 7.66 (1H, *d*, $J = 2$), 7.78 (2H, *d*, $J = 9$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.5 (CH_3), 56.6 (CH_3), 62.1 (CH_3), 113.9 (2CH), 116.3 (CH), 118.1 (CH), 129.0 (C), 132.3 (2CH), 133.3 (C), 143.9 (C), 145.7 (C), 154.1 (C), 163.6 (C), 192.3 (C). HRMS ($C_{16}H_{15}NO_6$ + H^+): calcd 318.0978 (M + H^+), found 318.0974.

(3-Amino-4,5-dimethoxyphenyl)(4-methoxyphenyl)methanone (40). 55 mg (0.17 mmol) of **39** were dissolved in CH_2Cl_2 (30 mL) then 70 mg (1.05 mmol) of zinc powder and acetic acid (two drops) were added to the solution at 0 °C. After 24 h stirring at room temperature, the reaction mixture was filtered through Celite, washed with brine, extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 47 mg (0.16 mmol, 95 %) of **40**. IR (KBr): 3364, 1640, 1594, 1353, 1250, 1172, 1126, 847 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.85 (3H, *s*), 3.87 (3H, *s*), 3.88 (3H, *s*), 6.76 (1H, *s*), 6.77 (1H, *s*), 7.93 (2H, *d*, $J = 8.8$), 7.81 (2H, *d*, $J = 8.8$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.3 (CH_3), 56.7 (CH_3), 60.8 (CH_3), 104.8 (CH), 111.9 (CH), 114.2 (2CH), 131.3 (C), 133.2 (2CH), 134.8 (C), 139.7 (C), 140.8 (C), 153.4 (C), 163.8 (C), 195.9 (C). HRMS ($C_{16}H_{17}NO_4$ + H^+): calcd 288.1238 (M + H^+), found 288.1234. HPLC: C_{18} t_R: 6.3 min.

Methyltriphenylphosphonium iodide (41). A solution of triphenylphosphine (37 g, 141.07 mmol) and iodomethane (8.8 mL, 141.25 mmol) in dry toluene (250 mL) was stirred at room temperature for 24 h. 52.2 g (14 mmol, 91 %) of the phosphonium salt **41** were obtained and used without further purification.

1,2-Dimethoxy-5-(1-(4-methoxyphenyl)vinyl)-3-nitrobenzene (42). 1.80 g (4.35 mmol) of **41** were dissolved in dry THF. The solution was cooled to −78 °C, then 2.3 mL (3.62 mmol) of *n*-BuLi 1.6 M in hexane was added. After 1 h stirring at −78 °C, ketone **39** (460 mg, 1.45 mmol) dissolved in THF was added to the solution. The reaction mixture was stirred for 24 h at room temperature, poured onto NH_4Cl, and extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated to dryness. The crude residue (1.25 g) was purified by flash column chromatography (hexane/EtOAc 8:2) to yield 327 mg (1.04 mmol, 72 %) of **42**. IR (KBr): 1606, 1531, 1458, 1361, 1253, 1179, 838 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.82 (3H, s), 3.85 (3H, s), 3.99 (3H, s), 5.36 (1H, s), 5.44 (1H, s), 6.87 (2H, d, J = 8.4), 7.04 (1H, d, J = 2.4), 7.24 (2H, d, J = 8.4), 7.30 (1H, d, J = 2.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.3 (CH_3), 56.4 (CH_3), 62.0 (CH_3), 113.8 (2CH), 114.3 (CH_2), 115.7 (CH), 115.8 (CH), 128.4 (C), 129.3 (2CH), 132.1 (C), 138.0 (C), 142.2 (C), 147.6 (C), 153.6 (C), 159.7 (C). HRMS ($C_{17}H_{17}NO_5$ + Na^+): calcd 338.1004 (M + Na^+), found 338.0999.

2,3-dimethoxy-5-(1-(4-methoxyphenyl)vinyl)aniline (43). 300 mg (0.95 mmol) of **42** were dissolved in CH_2Cl_2 (30 mL) then 375 mg (5.71 mmol) of zinc powder and acetic acid (600 μL, 10.47 mmol) were added to the solution. After 16 h stirring at room temperature, the reaction mixture was filtered through Celite and the solvent evaporated. The crude residue (360 mg) was purified by flash column chromatography (hexane/EtOAc 7:3) to yield 210 mg (0.74 mmol, 80 %) of **43**. IR (film): 3370, 1607, 1508, 1243, 1126, 839 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 4.10 (3H, *s*), 4.13 (3H, *s*), 4.18 (3H, *s*), 5.62 (1H, *d*, $J = 1.2$), 5.64 (1H, *d*, $J = 1.2$), 6.66 (1H, *d*, $J = 2.0$), 6.72 (1H, *d*, $J = 2.0$), 7.18 (2H, *d*, $J = 6.4$), 7.61 (2H, *d*, $J = 6.4$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.3 (CH_3), 55.7 (CH_3), 59.9 (CH_3), 103.1 (CH), 109.3 (CH_2), 112.2 (CH), 113.4 (2CH), 129.5 (2CH), 133.9 (C), 135.8 (C), 137.9 (C), 139.5 (C), 149.5 (C), 152.4 (C), 159.3 (C). HRMS ($C_{17}H_{19}NO_3$ + H^+): calcd 286.1443 (M + H^+), found 286.1440. HPLC: C_{18} t_R: 14.6 min.

***N,N*-dimetil-2,3-dimetoxi-5-(1-(4-metoxifenil)etenil)anilina (44).** A solution of **43** (90 mg, 0.32 mmol) and iodomethane (200 μL, 3.15 mmol) in acetone (5 mL) was stirred for 24 h at 70 °C. The reaction mixture was concentrated under vacuum to afford 98 mg (0.31 mmol, 97 %) of compound **44**. IR (film): 1606, 1510, 1343, 1247, 1177, 838 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 3.23 (6H, *s*), 3.76 (3H, *s*), 3.79 (3H, *s*), 4.12 (3H, *s*), 5.34 (1H, *s*), 5.38 (1H, *s*), 6.81 (2H, *d*, $J = 8.4$), 6.89 (1H, *s*), 7.15 (1H, *s*), 7.18 (2H, *d*, $J = 8.4$). ^{13}C NMR (100 MHz, CD_3OD): δ 47.3 ($2CH_3$), 55.4 (CH_3), 56.4 (CH_3), 62.9 (CH_3), 111.4 (CH), 113.8 (CH), 129.3 (CH_2), 132.4 (C), 133.5 (2CH), 138.9 (2C), 140.4 (C), 147.3 (2CH), 152.5 (2C), 159.6 (C). HRMS ($C_{19}H_{23}NO_3 + H^+$): calcd 314.1756 (M + H^+), found 314.1746. HPLC: C_{18} t_R: 20.1 min.

1-(Bromomethyl)-4-methoxybenzene (45). To a solution of (4-methoxyphenyl)methanol (4.9 mL, 39 mmol) in dry Et_2O (30 mL) at 40 °C, was added PBr_3 (3.7 mL, 39 mmol). The mixture was stirred at room temperature for 4 h. Then the reaction was treated with 5 % $NaHCO_3$ and extracted with CH_2Cl_2. The organics were dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 7.79 g (38.7 mmol, 98 %) of **45**. ^1H NMR (400 MHz, $CDCl_3$): δ 3.81 (3H, *s*), 4.51 (2H, *s*), 6.86 (2H, *d*, $J = 8.8$), 7.32 (2H, *d*, $J = 8.8$).

(4-Methoxybenzyl)triphenylphosphonium bromide (46). A solution of triphenylphosphine (10.2 g, 38.7 mmol) and **45** (7.79 g, 38.7 mmol) in dry toluene (250 mL) was stirred at room temperature for 15 h. The precipitate formed was filtered to afford 13.3 g (28.7 mmol, 75 %) of the phosphonium salt **46**, which was used without further purification. M.p.: 240 °C (benzene). ^1H NMR (400 MHz, $CDCl_3$): δ 3.73 (3H, *s*), 5.33 (2H, *d*, $J = 13.9$), 6.65 (2H, *d*, $J = 8.8$), 7.02 (2H, *d*, $J = 8.8$), 7.50–7.80 (15H, *m*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 29.9 (CH_2, *d*, $J = 45$), 54.9 (CH_3), 114.0 (2CH), 117.5 (3C, *d*, $J = 85$), 118.2 (C, *d*, $J = 9$), 129.9 (6CH, *d*, $J = 11$), 132.3 (2CH).

1,2-Dimethoxy-5-(4-methoxystyryl)-3-nitrobenzene (47). 4.80 g (10.36 mmol) of the phosphonium salt **46** was dissolved in dry THF (40 mL). The solution was cooled to −78 °C, then 5.3 mL (8.52 mmol) of *n*-BuLi 1.6 M in hexane was added. After 1 h stirring at −78 °C, the aldehyde **37** (1.20 g, 5.68 mmol) dissolved in dry THF was added to the solution. The reaction mixture was stirred for 24 h at room temperature, poured onto NH_4Cl, and extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford 4.85 g of a 1:2 mixture of **47(*Z* + *E*)**. Crude residue was purified by flash column chromatography (hexane/EtOAc 7:3) to yield 90 mg (0.28 mmol, 5 %) of **47(*Z*)**, 950 mg (3.01 mmol, 53 %) of **47(*E*)**, and 650 mg (2.06 mmol, 36 %) of **47(*Z* + *E*)**. **47(*Z*)**: ^1H NMR (400 MHz, $CDCl_3$): δ 3.65 (3H, *s*), 3.82 (3H, *s*), 3.83 (3H, *s*), 6.35 (1H, *d*, $J = 12.2$), 6.61 (1H, *d*, $J = 12.2$), 6.80 (2H, *d*, $J = 6.6$), 6.99 (1H, *d*, $J = 2.4$), 7.17 (2H, *d*, $J = 6.6$), 7.20 (1H, *d*, $J = 2.4$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.3 (CH_3), 56.1 (CH_3), 62.1 (CH_3), 113.8 (2CH), 116.2 (2CH), 116.4 (C), 126.3 (CH), 130.1 (2CH), 130.5 (C), 131.5 (CH), 141.4 (C), 153.4 (2C), 159.1 (C). **47(*E*)**: M.p.: 139–140 °C (CH_2Cl_2/Hexane). IR (film): 1600, 1508, 1236, 1127 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.84 (3H, *s*), 3.97 (3H, *s*), 3.98 (3H, *s*), 6.82 (1H, *d*, $J = 16$), 6.89 (2H, *d*, $J = 7$), 6.99 (1H, *d*, $J = 16$), 7.17 (1H, *d*, $J = 2$), 7.42 (2H, *d*, $J = 7$), 7.46 (1H, *d*, $J = 2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.3 (CH_3), 56.4 (CH_3), 62.0 (CH_3), 113.2 (CH), 113.5 (C), 114.0 (CH), 114.5 (2CH), 123.9 (CH), 127.9 (2CH), 127.9 (C), 130.2 (CH), 133.0 (C), 141.7 (C), 154.1 (C), 159.8 (C). HRMS ($C_{17}H_{17}NO_5$ + H^+): calcd 316.1185 (M + H^+), found 316.1184.

2,3-Dimethoxy-5-(4-methoxystyryl)aniline (48). 500 mg (1.58 mmol) of **47(*Z* + *E*)** were dissolved in CH_2Cl_2 (15 mL), then 630 mg (9.51 mmol) of zinc powder and acetic acid (2 mL) were added to the solution at 0 °C. After 4 h stirring at room temperature, the reaction mixture was filtered through Celite and the solvent evaporated to yield 836 mg of a 1:3 mixture of **48(*Z* + *E*)**. Crude residue was purified by flash column chromatography (hexane/EtOAc 1:1) to yield 88 mg (0.31 mmol, 20 %) of **48(*Z*)**, 154 mg (0.54 mmol, 34 %) of **48(*E*)**, and 200 mg (0.70 mmol, 44 %) of **48(*Z* + *E*)**. **48(*Z*)**: ^1H NMR (400 MHz, $CDCl_3$): δ 3.64 (3H, *s*), 3.78 (3H, *s*), 3.81 (3H, *s*), 6.30 (1H, *d*, $J = 12$), 6.32 (1H, *s*), 6.38 (1H, *s*), 6.44 (1H, *d*, $J = 12$), 6.77 (2H, *d*, $J = 8.6$), 7.24 (2H, *d*, $J = 8.6$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.7 (CH_3), 55.2 (CH_3), 59.5 (CH_3), 100.0 (CH), 106.4 (CH), 113.6 (2CH), 126.2 (CH), 126.8 (CH), 127.1 (2CH), 129.7 (C), 133.3 (C), 135.1 (C), 140.1 (C), 152.5 (C), 158.7 (C). **48(*E*)**: IR (KBr): 3470, 1604, 1507, 1245, 1125 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.82 (3H, *s*), 3.84 (3H, *s*), 3.89 (3H, *s*), 6.49 (1H, *d*, $J = 2$), 6.55 (1H, *d*, $J = 2$), 6.80 (1H, *d*, $J = 16.2$), 6.88 (2H, *d*, $J = 8.6$), 6.98

(1H, *d*, *J* = 16.2), 7.41 (1H, *d*, *J* = 8.6). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.6 ($2CH_3$), 60.0 (CH_3), 100.5 (CH), 106.8 (CH), 114.1 (2CH), 127.0 (2CH), 127.5 (2CH), 130.2 (C), 135.5 (2C), 152.9 (C), 159.1 (2C). HRMS ($C_{17}H_{19}NO_3 + H^+$): calcd 286.1443 ($M + H^+$), found 286.1427. HPLC: C_{18} t_R: 14.9 min, t_R: 15.6 min.

2,3-Dimethoxy-5-(4-methoxystyryl)-*N*,*N*-dimethylaniline (49). A solution of **48** (110 mg, 0.39 mmol) and iodomethane (1.2 mL, 19 mmol) in acetone (10 mL) was stirred for 24 h at 70 °C. The reaction mixture was concentrated under vacuum to afford 172 mg of a 1:3 mixture of **49(*Z* + *E*)**. Crude residue was purified by flash column chromatography (hexane/EtOAc 1:1) to yield 15 mg (0.05 mmol, 13 %) of **49(*Z*)**, 17 mg (0.06 mmol, 14 %) of **49(*E*)**, and 74 mg (0.24 mmol, 61 %) of **49(*Z* + *E*)** 1:1. **49(*Z*)**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.72 (6H, *s*), 3.65 (3H, *s*), 3.84 (3H, *s*), 3.90 (3H, *s*), 6.40 (1H, *d*, *J* = 12), 6.48 (1H, *d*, *J* = 12), 6.51 (2H, *s*), 6.79 (2H, *d*, *J* = 8.8), 7.24 (2H, *d*, *J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 42.9 ($2CH_3$), 55.2 (CH_3), 55.7 (CH_3), 59.5 (CH_3), 106.4 (CH), 111.7 (CH), 113.5 (2CH), 128.8 (C), 129.1 (2CH), 129.9 (C), 130.2 (2CH), 132.5 (C), 140.2 (C), 152.9 (C), 158.6 (C). HPLC: C_{18} t_R: 19.8 min. **49(*E*)**: IR (KBr): 1576, 1458, 1253, 1175, 1086 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.88 (6H, *s*), 3.80 (3H, *s*), 3.85 (3H, *s*), 3.91 (3H, *s*), 6.27 (1H, *d*, *J* = 16.4), 6.38 (1H, *d*, *J* = 16.4), 6.48 (1H, *d*, *J* = 16.4), 6.75 (2H, *d*, *J* = 8.6), 6.85 (1H, *d*, *J* = 16.4), 7.47 (2H, *d*, *J* = 8.6). ^{13}C NMR (100 MHz, $CDCl_3$): δ 43.0 ($2CH_3$), 55.3 (CH_3), 56.1 (CH_3), 59.5 (CH_3), 103.3 (CH), 109.2 (CH), 114.1 (2CH), 126.9 (C), 129.1 (2CH), 127.6 (2CH), 130.0 (C), 133.1 (C), 140.6 (C), 153.5 (C), 159.2 (C). HRMS ($C_{19}H_{23}NO_3 + Na^+$): calcd 336.1576 ($M + Na^+$), found 336.1564.

(*Z*)-2,3-dimethoxy-5-(4-methoxystyryl)-*N*,*N*,*N*-trimethylbenzenaminium iodide (50). A solution of a mixture of **49(*Z* + *E*)** (200 mg, 0.63 mmol), iodomethane (600 μL, 9.25 mmol) and $AgNO_3$ (320 mg, 1.85 mmol) in acetone (15 mL) was stirred for 24 h at room temperature. The reaction mixture was filtered through Celite and concentrated under vacuum to afford 100 mg (0.31 mmol, 49 %) of **50(*Z*)**. IR (film): 1603, 1508, 1459, 1252, 1177, 1060 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.59 (9H, *s*), 3.67 (3H, *s*), 3.70 (3H, *s*), 3.74 (3H, *s*), 6.50 (1H, *d*, *J* = 12), 6.70 (1H, *d*, *J* = 12), 6.87 (2H, *d*, *J* = 8.4), 7.15 (1H, *s*), 7.18 (1H, *s*), 7.21 (2H, *d*, *J* = 8.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.5 (CH_3), 57.6 (CH_3), 58.0 ($3CH_3$), 61.8 (CH_3), 113.4 (CH), 113.9 (2CH), 115.2 (CH), 127.1 (CH), 128.8 (C), 130.1 (2CH), 131.7 (CH), 133.5 (C), 137.8 (C), 140.8 (C), 153.7 (C), 159.0 (C). HRMS ($C_{20}H_{25}NO_3 + H^+$): calcd 328.1913 ($M + H^+$), found 328.1911. HPLC: C_{18} t_R: 7.1 min.

2-(3,4-Dimethoxy-5-nitrophenyl)-1,3-dioxolane (51). To a solution of 8.50 g (40.2 mmol) of the aldehyde **37** in dry toluene (200 mL), ethylene glycol (7 mL, 125 mmol) and *p*-toluenesulfonic acid (1.10 g, 6.40 mmol) were added. After 24 h, $NaHCO_3$ was added and the solvent was evaporated. Then, the residue was extracted with EtOAc, washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford 9.88 g (38.7 mmol, 96 %) of **51**. IR (film): 1608, 1537, 1287, 1149 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.92 (3H, *s*), 3.97 (3H, *s*), 4.02 (2H, *m*), 4.08 (2H, *m*), 5.75 (1H, *s*), 7.20 (1H, *d*, *J* = 2), 7.42 (1H, *d*, *J* = 2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.4 (CH_3), 62.2 (CH_3), 65.3 ($2CH_2$), 102.1 (CH), 113.7 (CH), 114.0 (CH), 119.3 (C), 134.2 (C), 143.1 (C), 154.1 (C).

5-(1,3-Dioxolan-2-yl)-2,3-dimethoxyaniline (52). 500 mg (1.96 mmol) of **51** were dissolved in CH_2Cl_2 (20 mL), then 770 mg (11.75 mmol) of zinc powder and acetic acid (drops) were added to the solution. After 15 h stirring at room temperature, the reaction mixture was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford 404 mg (1.79 mmol, 92 %) of **52**. IR (film): 3368, 1597, 1457, 1350, 1232, 1143 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.79 (3H, *s*), 3.84 (3H, *s*), 4.00 (2H, *m*), 4.09 (2H, *m*), 5.72 (1H, *s*), 6.46 (1H, *d*, *J* = 2), 6.51 (1H, *d*, *J* = 2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.4 (CH_3), 60.5 (CH_3), 65.9 ($2CH_2$), 100.7 (CH), 104.4 (CH), 107.7 (CH), 130.7 (C), 134.5 (C), 141.4 (C), 153.6 (C).

3-Amino-4,5-dimethoxybenzaldehyde (53). Compound **52** (320 mg, 1.42 mmol) was dissolved in methanol (6 mL). A mixture of H_2O/HCl(c) (drops) was added to the solution and stirred for 4 h. The reaction mixture was washed with brine and extracted with EtOAc. The organics were dried and evaporated to dryness to afford 250 mg (1.38 mmol, 97 %) of **53**. IR (film): 3362, 1688, 1582, 1458, 1088 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.87 (3H, *s*), 3.89 (3H, *s*), 6.92 (1H, *d*, *J* = 2), 6.97 (1H, *d*, *J* = 2), 9.78 (1H, *s*).

***N*-(5-formyl-2,3-dimethoxyphenyl)acetamide (54).** 250 mg (1.38 mmol, 97 %) of **53** were dissolved in CH_2Cl_2 (20 mL). Acetic anhydride (260 μL, 2.76 mmol) and pyridine (230 μL, 2.77 mmol) were added to the solution. After 2 h stirring at room temperature, the reaction mixture was poured onto brine and extracted with CH_2Cl_2. The organics were dried and evaporated to dryness to afford 223 mg (1.00 mmol, 72 %) of **54**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.21 (3H, *s*), 3.85 (3H, *s*), 3.95 (3H, *s*), 7.20 (1H, *s*), 8.52 (1H, *s*), 9.84 (1H, *s*).

***N*-(2,3-dimethoxy-5-(4-methoxystyryl)phenyl)acetamide (55).** 950 mg (2.04 mmol) of the phosphonium salt **46** were dissolved in dry THF (30 mL). The solution was cooled to −78 °C, then 1.1 mL (1.61 mmol) of *n*-BuLi 1.6 M in hexane was added. After 1 h stirring at −78 °C, the aldehyde **54** (240 mg, 1.07 mmol) dissolved in dry THF was added to the solution. The reaction mixture was stirred for 24 h at room temperature, poured onto NH_4Cl, and extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford 926 mg of a 1:3 mixture of **55(*Z* + *E*)**. Crude residue was purified by flash column chromatography (hexane/EtOAc 7:3) to yield 20 mg (0.06 mmol, 6 %) of **55(*Z*)**, 33 mg (0.10 mmol, 10 %) of **55(*E*)**, and 137 mg (0.42 mmol, 39 %) of **55(*Z* + *E*)** 1:1. **55(*Z*)**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.19 (3H, *s*), 3.55 (3H, *s*), 3.78 (3H, *s*), 3.89 (3H, *s*), 6.45 (1H, *d*, *J* = 12), 6.49 (1H, *d*, *J* = 12), 6.59 (1H, *d*, *J* = 1.6), 6,78 (2H, *d*, *J* = 9.2), 7.21 (2H, *d*, *J* = 9.2), 7.90 (1H, *d*, *J* = 1.6). ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.0 (CH_3), 55.2 (CH_3), 55.5 (CH_3), 60.8 (CH_3), 107.7 (CH), 113.5 (2CH), 114.1 (CH), 127.6 (C), 128.8 (CH), 129.5 (CH), 129.7 (C), 130.2 (2CH), 131.7 (C), 133.5 (C), 151.1 (C), 158.6 (C), 168.2 (C). HPLC: C_{18} t_R: 14.8 min. **55(*E*)**: IR (film): 3326, 1684, 1591, 1248, 1105 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.22 (3H, *s*), 3.84 (3H, *s*), 3.87 (3H, *s*), 3.91 (3H, *s*), 6.78 (1H, *d*, *J* = 1.6), 6.87 (2H, *d*, *J* = 8.8), 6.89 (1H, *d*, *J* = 16), 6.97 (1H, *d*, *J* = 16), 7.42 (2H, *d*, *J* = 8.8), 8.17 (1H, *d*, *J* = 1.6). HRMS ($C_{19}H_{21}NO_4 + H^+$): calcd 328.1549 ($M + H^+$), found 328.1543.

***N*-(2,3-dimethoxy-5-(4-methoxystyryl)phenyl)-*N*-methylacetamide (56).** To a stirred solution at 0 °C of **55(*Z* + *E*)** (140 mg, 0.42 mmol) in dry THF (10 mL), sodium hydride (31 mg, 1.28 mmol) and iodomethane (140 μL, 2.13 mmol) were added. After 15 h at room temperature, the solvent was removed under reduced pressure. The residue was dissolved in EtOAc and washed with brine, dried (Na_2SO_4), and concentrated in a vacuum to afford 145 mg of a 2:1 mixture of **56(*Z* + *E*)**. Crude residue was purified by flash column chromatography (hexane/EtOAc 6:4) to yield 60 mg (0.18 mmol, 43 %) of **56(*Z*)** and 44 mg (0.13 mmol, 32 %) of **56(*Z* + *E*)**. **56(*Z*)**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.81 (3H, *s*), 3.13 (3H, *s*), 3.72 (3H, *s*), 3.78 (3H, *s*), 3.81 (3H, *s*), 6.38 (1H, *d*, *J* = 12), 6.54 (1H, *d*, *J* = 12), 6.64 (1H, *d*, *J* = 2), 6.75 (2H, *d*, *J* = 8.8), 6.81 (1H, *d*, *J* = 2), 7.17 (2H, *d*, *J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.4 (CH_3), 36.1 (CH_3), 54.8 (CH_3), 55.4 (CH_3), 60.6 (CH_3), 111.9 (CH), 113.3 (2CH), 120.6 (CH), 127.0 (CH), 128.8 (C), 129.7 (2CH), 130.0 (CH), 133.3 (C), 137.2 (C), 143.3 (C), 152.7 (C), 158.5 (C), 170.6 (C). HPLC: C_{18} t_R: 17.4 min. **56(*E*)**: IR (film): 1661, 1596, 1572, 1511, 1254, 1063 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.87 (3H, *s*), 3.21 (3H, *s*), 3.72 (3H, *s*), 3.78 (3H, *s*), 3.81 (3H, *s*), 6.85 (1H, *d*, *J* = 16), 6.86 (2H, *d*, *J* = 8.8), 6.87 (1H, *d*, *J* = 2.4), 6.94 (1H, *d*, *J* = 16) 7.00 (1H, *d*, *J* = 2.4), 7.40 (2H, *d*, *J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.8 (CH_3), 36.6 (CH_3), 55.3 (CH_3), 56.0 (CH_3), 61.1 (CH_3), 109.3 (CH), 114.2 (2CH), 125.1 (CH), 127.4 (2CH), 129.5 (C), 130.4 (2CH), 134.2 (C), 138.0 (C), 143.7 (C), 153.1 (C), 159.5 (C), 171.0 (C). HRMS ($C_{20}H_{23}NO_4 + Na^+$): calcd 364.1525 ($M + Na^+$), found 364.1522.

***Tert*-butyl (5-(1,3-dioxolan-2-yl)-2,3-dimethoxyphenyl)carbamate (57a) and *tert*-butyl (5-formyl-2,3-dimethoxyphenyl)carbamate (57b).** Boc_2O (4.10 g, 18.65 mmol) was added to a stirred solution of **52** (2.80 g, 12.43 mmol) in dry THF (60 mL). After 48 h stirring at

reflux, the solvent was removed under reduced pressure and the residue was dissolved in EtOAc and washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue (3.20 g) was purified by flash column chromatography (hexane/EtOAc 8:2) to afford compounds **57a** (2.10 g, 6.46 mmol, 52 %) and **57b** (1.03 g, 3.66 mmol, 29 %). **(57a)**: M.p.: 97–98 °C (CH_2Cl_2). IR (KBr): 3425, 1731, 1533, 1240, 1152, 1002 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.52 (9H, *s*), 3.84 (3H, *s*), 3.88 (3H, *s*), 4.01 (2H, *m*), 4.13 (2H, *m*), 5.72 (1H, *s*), 6.75 (1H, *d*, J = 2), 7.17 (1H, *d*, J = 2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.2 ($3CH_3$), 55.7 (CH_3), 60.5 (CH_3), 65.2 ($2CH_2$), 80.4 (C), 103.6 (CH), 103.7 (CH), 109.2 (CH), 132.4 (C), 133.7 (C), 137.1 (C), 151.9 (C), 152.4 (C). **(57b)**: IR (film): 3432, 1727, 1604, 1534, 1242, 1156, 1048 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.54 (9H, *s*), 3.92 (3H, *s*), 3.95 (3H, *s*), 7.17 (1H, *d*, J = 1.6), 7.20 (1H, *d*, J = 1.6), 9.88 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.8 ($3CH_3$), 56.4 (CH_3), 61.3 (CH_3), 81.6 (C), 104.4 (CH), 116.4 (CH), 132.7 (C), 133.9 (C), 141.9 (C), 152.9 (C), 153.0 (C), 192.2 (CH).

***Tert*-butyl (5-formyl-2,3-dimethoxyphenyl)(methyl)carbamate (58).** To a stirred solution at 0 °C of **57b** (1.03 g, 3.66 mmol) in dry THF (30 mL), sodium hydride (266 mg, 10.98 mmol) and iodomethane (1.2 mL, 18.3 mmol) were added. After 15 h at room temperature, the solvent was removed under reduced pressure. The residue was dissolved in EtOAc and washed with brine, dried (Na_2SO_4), and concentrated in a vacuum to afford 967 mg (3.28 mmol, 90 %) of **58**. IR (KBr): 1730, 1697, 1582, 1256, 1152, 1073 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.25 (9H, *s*), 2.91 (3H, *s*), 3.88 (3H, *s*), 3.91 (3H, *s*), 6.81 (1H, *d*, J = 1.6), 6.87 (1H, *d*, J = 1.6), 9.85 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.1 (CH_3), 28.6 ($3CH_3$), 56.5 (CH_3), 61.1 (CH_3), 80.7 (C), 109.6 (CH), 125.7 (CH), 130.0 (C), 132.1 (C), 137.8 (C), 154.2 (C), 156.0 (C), 190.9 (CH).

***Tert*-butyl (2,3-dimethoxy-5-(4-methoxystyryl)phenyl)(methyl) carbamate (59).** 630 mg (1.36 mmol) of the phosphonium salt **46** were dissolved in dry THF (20 mL). The solution was cooled to −78 °C, then 640 μL (1.02 mmol) of *n*-BuLi 1.6 M in hexane was added. After 1 h stirring at −78 °C, the aldehyde **58** (200 mg, 0.67 mmol) dissolved in dry THF (10 mL) was added to the solution. The reaction mixture was stirred for 24 h at room temperature, poured onto NH_4Cl, and extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford 930 mg of a 1:2 mixture of **59(*Z* + *E*)**. Crude residue was purified by flash column chromatography (hexane/EtOAc 8:2) to yield 76 mg (0.19 mmol, 28 %) of **59(*Z*)**, 142 mg (0.35 mmol, 53 %) of **59(*E*)**, and 42 mg (0.10 mmol, 16 %) of **59(*Z* + *E*)** 3:1. **59(*Z*)**: ^1H NMR (400 MHz, $CDCl_3$): δ 1.43 (9H, *s*), 3.07 (3H, *s*), 3.64 (3H, *s*), 3.76 (3H, *s*), 3.80 (3H, *s*), 6.36 (1H, *d*, J = 12), 6.46 (1H, *d*, J = 12), 6.72 (1H, *d*, J = 2), 6.75 (2H, *d*, J = 8), 7.77 (1H, *d*, J = 2), 7.21 (2H, *d*, J = 8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 27.9 (CH_3), 28.3 ($3CH_3$), 55.2 (CH_3), 55.7 (CH_3), 60.6 (CH_3), 79.8 (C), 111.4 (CH), 113.5 (2CH), 114.3 (CH), 127.8 (C), 128.0 (CH), 129.4 (CH), 129.6 (C), 130.1 (2CH), 132.8 (C), 137.0 (C), 152.7 (C), 155.1 (C), 158.7 (C). HPLC: C_{18} t_R: 21.1 min. **59(*E*)**: IR (KBr): 1700, 1606, 1511, 1367, 1254, 1153 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.40 (9H, *s*), 3.15 (3H, *s*), 3.81 (6H, *s*), 3.89 (3H, *s*), 6.85 (1H, *d*, J = 16.4), 6.88 (2H, *d*, J = 8.4), 6.90 (1H, *d*, J = 2), 6.93 (1H, *d*, J = 16.4), 6.65 (1H, *d*, J = 2), 7.42 (2H, *d*, J = 8.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.1 (CH_3), 28.1 ($3CH_3$), 55.1 (CH_3), 55.7 (CH_3), 60.5 (CH_3), 79.7 (C), 108.1 (CH), 113.3 (CH), 113.9 (2CH), 118.8 (C), 125.6 (CH), 127.4 (2CH), 127.6 (CH), 129.7 (C), 130.0 (C), 137.2 (C), 152.7 (C), 155.0 (C), 159.1 (C). HRMS ($C_{23}H_{29}NO_5$ + H^+): calcd 400.2124 (M + H^+), found 400.2123.

(*Z*)-2,3-dimethoxy-5-(4-methoxystyryl)-*N*-methylaniline (60). To a solution of **59(*Z*)** (70 mg, 0.17 mmol) in dry methanol (3 mL), trimethylsilyl chloride (67 μL, 0.53 mmol) was added. After 24 stirring at room temperature, the solution was concentrated in vacuo and re-dissolved with EtOAc. Then, it was treated with 5 % $NaHCO_3$, washed with brine, dried over Na_2SO_4, and evaporated to dryness to give 100 mg of a 6:4 mixture of **60(*Z* + *E*)**. Crude residue was purified by flash column chromatography (hexane/EtOAc 8:2) to yield 25 mg (0.08 mmol, 49 %) of **60(*Z*)**, and 20 mg (0.06 mmol, 39 %) of **60(*Z* + *E*)** 3:1. IR (KBr): 3387, 1590, 1512, 1459, 1254, 1176 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 2.61 (3H, *s*), 3.55 (3H, *s*), 3.68 (6H, *s*), 6.15 (1H, *d*, J = 2.8), 6.33 (1H, *d*, J = 12), 6.35 (1H, *d*, J = 2.8), 6.37 (1H, *d*, J = 12), 6.67 (2H, *d*, J = 8.4), 7.16 (2H, *d*, J = 8.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.4 (CH_3), 55.2 (CH_3), 55.7 (CH_3), 60.0 (CH_3), 102.0 (CH), 104.5 (CH), 113.4 (2CH), 128.8 (CH), 129.4 (CH), 130.0 (C), 130.3 (2CH), 133.5 (C), 134.3 (C), 143.0 (C), 151.7 (C), 158.5 (C). HRMS ($C_{18}H_{21}NO_3$ + H^+): calcd 300.1600 (M + H^+), found 300.1583. HPLC: C_{18} t_R: 18.2 min.

(4-(Dimethylamino)phenyl)methanol (61). To a solution at 0 °C of 4-(dimethylamino)benzaldehyde (3 g, 20.1 mmol) in methanol (20 mL), $NaBH_4$ (761 mg, 20.1 mmol) was added. After 2 h, the reaction mixture was concentrated under reduced pressure, diluted with EtOAc, washed with brine, dried (Na_2SO_4), filtered, and concentrated in vacuo to give 2.90 g (19.2 mmol, 95 %) of **61**. IR (KBr): 3385, 2870, 1521, 1225 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 2.95 (6H, *s*), 4.53 (2H, *s*), 6.77 (2H, *d*, J = 8.8), 7.26 (2H, *d*, J = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 41.3 ($2CH_3$), 65.0 (CH_2), 113.4 (2CH), 129.1 (2CH), 130.0 (C), 150.6 (C).

(4-(Dimethylamino)benzyl)triphenylphosphonium chloride (62b). Compound **61** (1.60 g, 10.6 mmol) was dissolved in CH_2Cl_2 and a stream of HCl (g), generated from the treatment of NaCl with H_2SO_4, was bubbled. The reaction mixture turned yellow, indicating the formation of 4-(chloromethyl)-*N*,*N*-dimethylaniline (**62a**). Then, triphenylphosphine (3.4 g, 12.7 mmol) dissolved in CH_2Cl_2 (15 mL) was added to the mixture and stirred for 24 h. The solvent was evaporated and the residue was re-crystallized from toluene to afford the phosphonium salt **62b** (2.72 g, 6.30 mmol, 60 %). ^1H NMR (400 MHz, $CDCl_3$): δ 3.14 (6H, *s*), 5.57 (1H, *s*), 5.65 (1H, *s*), 7.33–7.78 (19H, *m*).

4-(3,4-Dimethoxy-5-nitrostyryl)-*N*,*N*-dimethylaniline (63). 2.70 g (5.64 mmol) of the phosphonium salt **62** was dissolved in dry THF (50 mL). The solution was cooled to −78 °C, then 3.3 mL (5.23 mmol) of *n*-BuLi 1.6 M in hexane were added. After 1 h stirring at −78 °C, the aldehyde **37** (850 mg, 4.03 mmol) dissolved in dry THF was added to the solution. The reaction mixture was stirred for 24 h at room temperature, poured onto NH_4Cl, and extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford 1.80 g of a 1:3 mixture of **63(*Z* + *E*)**. The crude residue was purified by flash column chromatography (hexane/EtOAc 9:1) to yield 163 mg (0.50 mmol, 12 %) of **63(*Z*)** and 464 mg (1.41 mmol, 35 %) of **63(*E*)**. **63(*Z*)**: ^1H NMR (400 MHz, $CDCl_3$): δ 2.92 (6H, *s*), 3.67 (3H, *s*), 3.94 (3H, *s*), 6.24 (1H, *d*, J = 12.4), 6.55 (1H, *d*, J = 12.4), 6.59 (2H, *d*, J = 8.8), 7.08 (1H, *d*, J = 2), 7.13 (2H, *d*, J = 8.8), 7.26 (1H, *d*, J = 2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.3 ($2CH_3$), 56.1 (CH_3), 62.0 (CH_3), 111.9 (2CH), 116.2 (2CH), 123.9 (C), 124.1 (CH), 129.9 (2CH), 132.2 (CH), 134.2 (C), 141.2 (C), 144.8 (C), 149.9 (C), 153.4 (C). **63(*E*)**: ^1H NMR (400 MHz, $CDCl_3$): δ 2.95 (6H, *s*), 3.69 (3H, *s*), 3.97 (3H, *s*), 6.70 (2H, *d*, J = 8.8), 6.78 (1H, *d*, J = 16), 6.98 (1H, *d*, J = 16), 7.16 (1H, *s*), 7.39 (2H, *d*, J = 8.8), 7.42 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 39.5 ($2CH_3$), 55.6 (CH_3), 61.2 (CH_3), 111.5 (2CH), 112.1 (CH), 120.7 (2CH), 123.7 (C), 127.1 (2CH), 130.0 (CH), 133.9 (C), 140.3 (C), 144.2 (C), 149.7 (C), 153.3 (C).

4-(3-Amino-4,5-dimethoxystyryl)-*N*,*N*-dimethylaniline (64). 120 mg (0.36 mmol) of **63(*Z*)** were dissolved in CH_2Cl_2 (15 mL), then 145 mg (2.22 mmol) of zinc powder and acetic acid (drops) were added to the solution at 0 °C. After16 h stirring at room temperature, the reaction mixture was filtered through Celite and the solvent evaporated to yield 120 mg of a 3:1 mixture of **64(*Z* + *E*)**. The crude residue was purified by flash column chromatography (hexane/EtOAc 7:3) to yield 63 mg (0.21 mmol, 59 %) of **64(*Z*)** and 11 mg (0.04 mmol, 10 %) of **64(*E*)**. **64(*Z*)**: ^1H NMR (400 MHz, $CDCl_3$): δ 2.93 (6H, *s*), 3.68 (3H, *s*), 3.83 (3H, *s*), 6.27 (1H, *d*, J = 12), 6.34 (1H, *d*, J = 2), 6.38 (1H, *d*, J = 2), 6.39 (1H, *d*, J = 12), 6.63 (2H, *d*, J = 8.8), 7.22 (2H, *d*, J = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.7 ($2CH_3$), 55.6 (CH_3), 59.9 (CH_3), 100.3 (CH), 106.6 (CH), 112.8 (2CH), 124.8 (C), 127.2 (2CH), 127.4 (2CH), 134.3 (C), 135.0 (C), 140.5 (C), 149.3 (C), 153.0 (C). **64(*E*)**: IR (film): 3365, 1607, 1517, 1231, 1128 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 2.98 (6H, *s*), 3.68 (3H, *s*), 3.89 (3H, *s*), 6.48 (1H, *d*, J = 2), 6.53 (1H, *d*, J = 2), 6.74 (2H, *d*, J = 6.8), 6.76 (1H, *d*, J = 16), 6.89 (1H, *d*, J = 16), 7.38 (2H, *d*, J

= 6.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.7 ($2CH_3$), 55.7 (CH_3), 60.0 (CH_3), 103.1 (CH), 109.2 (CH), 112.7 (2CH), 127.8 (C), 129.5 (2CH), 130.1 (2CH), 134.0 (C), 134.8 (C), 140.1 (C), 149.2 (C), 152.5 (C). HRMS ($C_{18}H_{22}N_2O_2 + H^+$): calcd 299.1760 ($M + H^+$), found 299.1758. HPLC: C_{18} t_R: 15.9 min.

***Tert*-butyl (5-(hydroxy(1-methyl-1*H*-indol-5-yl)methyl)-2,3-dimethoxyphenyl)(methyl) carbamate (65).** 1.30 g (5.72 mmol) of 5-bromo-1-methyl-1*H*-indole were dissolved in dry THF (30 mL). The solution was cooled to −78 °C, then 3.1 mL (4.85 mmol) of *n*-BuLi 1.6 M in hexane was added. After 1 h stirring at −78 °C, the aldehyde **58** (1.20 g, 4.40 mmol) dissolved in dry THF was added to the solution. The reaction mixture was stirred for 24 h at room temperature, poured onto NH_4Cl, and extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated to dryness. The crude residue (2.02 g) was purified by flash column chromatography (hexane/EtOAc 7:3) to yield 420 mg (0.98 mmol, 22 %) of **65**. IR (KBr): 3419, 1697, 1590, 1367, 1256, 1152 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.47 (9H, *s*), 3.16 (3H, *s*), 3.76 (3H, *s*), 3.82 (6H, *s*), 5.73 (1H, *s*), 6.58 (1H, *d*, *J* = 3.2), 6.96 (1H, *d*, *J* = 2), 7.14 (1H, *d*, *J* = 3.2), 7.38 (1H, *d*, *J* = 2), 7.39 (1H, *d*, *J* = 8.4), 7.77 (1H, *d*, *J* = 8.4), 8.11 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.7 ($3CH_3$), 33.4 (CH_3), 37.1 (CH_3), 56.5 (CH_3), 61.1 (CH_3), 65.7 (CH), 80.4 (C), 103.2 (CH), 108.9 (CH), 113.8 (CH), 119.5 (CH), 124.1 (C), 124.5 (CH), 125.4 (CH), 127.9 (C), 129.5 (C), 130.3 (C), 130.8 (CH), 132.1 (C), 137.5 (C), 153.4 (C), 155.4 (C).

***Tert*-butyl (2,3-dimethoxy-5-(1-methyl-1*H*-indole-5-carbonyl) phenyl)(methyl)carbamate (66).** Compound **65** (300 mg, 0.71 mmol) was dissolved in CH_2Cl_2 (20 mL) and cooled to 0 °C. PDC (318 mg, 0.84 mmol) was added and stirred at room temperature for 4 h. The reaction mixture was washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. Crude residue (352 mg) was purified by flash column chromatography (hexane/EtOAc 7:3) to yield 250 mg (0.59 mmol, 83 %) of pure product **66**. IR (KBr): 1694, 1644, 1581, 1253, 1154, 1073 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.43 (9H, *s*), 3.13 (3H, *s*), 3.74 (3H, *s*), 3.84 (6H, *s*), 6.57 (1H, *d*, *J* = 3.2), 7.14 (1H, *d*, *J* = 3.2), 7.24 (1H, *d*, *J* = 2), 7.38 (1H, *d*, *J* = 8.8), 7.39 (1H, *d*, *J* = 2), 7.76 (1H, *d*, *J* = 8.8), 8.11 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.3 ($3CH_3$), 30.2 (CH_3), 33.1 (CH_3), 56.1 (CH_3), 60.7 (CH_3), 80.1 (C), 102.8 (CH), 108.8 (CH), 112.1 (CH), 123.7 (CH), 123.9 (C), 124.2 (CH), 125.2 (CH), 127.6 (C), 129.1 (C), 130.3 (C), 130.5 (CH), 133.9 (C), 138.8 (C), 153.1 (C), 154.9 (C), 195.8 (C). HRMS ($C_{24}H_{28}N_2O_5 + H^+$): calcd 425.2076 ($M + H^+$), found 425.2076.

(3,4-Dimethoxy-5-(methylamino)phenyl)(1-methyl-1*H*-indol-5-yl)methanone (67). Ketone **66** (45 mg, 0.11 mmol) was dissolved in methanol (4 mL) and trimethylsilyl chloride (41 μL, 0.33 mmol) was added to the solution. After 24 h stirring at room temperature, the reaction mixture was concentrated under a vacuum, re-dissolved in CH_2Cl_2, washed with $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated to dryness. The crude residue (48 mg) was purified by flash column chromatography (hexane/EtOAc 6:4) to yield 20 mg (0.06 mmol, 56 %) of pure product **67**. IR (film): 3416, 1642, 1590, 1168, 1129 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.84 (3H, *s*), 3.85 (3H, *s*), 3.85 (3H, *s*), 3.88 (3H, *s*), 6.59 (1H, *d*, *J* = 3.2), 6.76 (1H, *d*, *J* = 1.6), 6.81 (1H, *d*, *J* = 1.6), 7.13 (1H, *d*, *J* = 3.2), 7.38 (1H, *dd*, *J* = 8.4), 7.82 (1H, *dd*, *J* = 8.4 and 1.6), 8.17 (1H, *d*, *J* = 1.6). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.9 (CH_3), 33.3 (CH_3), 56.3 (CH_3), 60.7 (CH_3), 101.7 (CH), 104.1 (CH), 106.7 (CH), 109.2 (CH), 120.4 (C), 124.2 (CH), 124.5 (CH), 126.8 (C), 128.2 (CH), 129.3 (C), 135.4 (C), 139.9 (C), 143.2 (C), 151.9 (C), 197.5 (C). HRMS ($C_{19}H_{20}N_2O_3 + H^+$): calcd 325.1552 ($M + H^+$), found 325.1552. HPLC: C_{18} t_R: 15.1 min.

***Tert*-butyl (2,3-dimethoxy-5-(1-(1-methyl-1*H*-indol-5-yl)vinyl) phenyl)(methyl)carbamate (68).** 1.70 g (4.24 mmol) of the phosphonium salt **41** was dissolved in dry THF (30 mL). The solution was cooled to −78 °C, then 2.2 mL (3.53 mmol) of *n*-BuLi 1.6 M in hexane were added. After 1 h stirring at −78 °C, ketone **66** (600 mg, 1.41 mmol) dissolved in THF was added to the solution. The reaction mixture was stirred for 24 h at room temperature, poured onto NH_4Cl, and extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated to dryness. The crude residue (1.10 g) was purified by flash column chromatography (hexane/EtOAc 7:3) to yield 278 mg (0.66 mmol, 47 %) of **68**. IR (film): 1699, 1577, 1495, 1246, 1153, 1075 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.40 (9H, *s*), 3.14 (3H, *s*), 3.81 (6H, *s*), 3.85 (3H, *s*), 5.36 (1H, *s*), 5.42 (1H, *s*), 6.46 (1H, *d*, *J* = 3.2), 6.78 (1H, *d*, *J* = 2), 6.86 (1H, *d*, *J* = 2), 7.06 (1H, *d*, *J* = 3.2), 7.22 (1H, *d*, *J* = 8.4), 7.27 (1H, *d*, *J* = 8.4), 7.60 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.7 ($3CH_3$), 30.8 (CH_3), 33.1 (CH_3), 56.3 (CH_3), 60.7 (CH_3), 80.1 (C), 101.7 (CH), 109.1 (CH), 111.4 (CH), 112.9 (CH_2), 121.2 (CH), 121.6 (CH), 122.7 (CH), 128.7 (2C), 129.6 (C), 129.8 (CH), 132.9 (C), 136.8 (C), 137.2 (C), 150.7 (C), 153.1 (C), 155.5 (C). HRMS ($C_{25}H_{30}N_2O_4 + H^+$): calcd 423.2284 ($M + H^+$), found 423.2274. HPLC: C_{18} t_R: 21.6 min.

4.1.3. Determination of aqueous solubility

The aqueous solubility was determined in a Helios Alfa Spectrophotometer using an approach based on the saturation shake-flask method. 1–2 mg of each selected compound were suspended in 300 μL pH 7.0 buffer and stirred for 72 h at room temperature. The resulting mixture was filtrated over a 45 μm filter to discard the insoluble residues. A scan between 270 and 400 nm for the tested compound was performed and the three maximum wavelengths of absorbance were selected. Calibration lines were performed at these wavelengths and the concentration in the supernatant was measured by UV absorbance. The final value was given as the average of the three measurements.

4.2. Biology

4.2.1. Cell lines and culture conditions

Cell lines MCF7 (human breast carcinoma), HeLa (human cervical carcinoma), and HEK-293 (human embryonic kidney) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10 % (v/v) heat-inactivated fetal bovine serum (HIFBS) (Lonza-Cambrex), 2 mM L-glutamine (Lonza-Cambrex, Karlskoga, Sweden) and 100 μg/mL streptomycin-100 IU/mL penicillin (Lonza-Cambrex) at 37 °C in humidified 95 % air and 5 % CO_2. Cell line HT-29 (human colon carcinoma) was cultured in RPMI 1640 medium (Gibco) supplemented with 10 % HIFBS and 100 μg/mL streptomycin-100 IU/mL penicillin at 37 °C in humidified 95 % air and 5 % CO_2 atmosphere. The presence of mycoplasma was routinely checked with the MycoAlert kit (Lonza) and only mycoplasma-free cells were used in the experiments.

4.2.2. Cell growth inhibition assay

To determine cell growth, cells in the exponential growth phase were seeded (100 μL/well in 96-well plates) with appropriate cell line concentration ($1.5{\cdot}10^4$ cells/mL for MCF7, HeLa, and HEK-293 and $3{\cdot}10^4$ cells/mL for HT-29) in complete DMEM or RPMI 1640 medium (see above) at 37 °C and 5 % CO_2 atmosphere. After 24 h incubation, to allow cells to attach to the plates, all compounds were added at 1 μM concentration (10 μL/well in 96-well plates) and the effect on the proliferation was evaluated 72 h post-treatment. Compounds showing antiproliferative effects at tested concentration were selected for IC_{50} calculation (concentration reducing proliferation to 50 % of the untreated controls) from 10^{-5} to 10^{-10} M concentration. Nonlinear curves fitting the experimental data were carried out for each compound. MCF7, HeLa, HT-29, and HEK-293 cell growth, when treated with the corresponding compound, were determined using the XTT (sodium 3′-[1 (phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)-benzenesulfonic acid hydrate) cell proliferation kit (Roche Molecular Biochemicals, Mannheim, Germany). A freshly prepared mixture solution of XTT labeling reagent with 0.02 % (v/v) PMS (*N*-methyl-dibenzopyrazine methyl sulfate) electron coupling reagent was added to cells (50 μL/well in 96-well plates, the total volume of 160 μL/well) and were incubated during the corresponding time according to each cell line (4 h for MCF7 and HeLa and 6 h for HT-29 and HEK-293), in a humidified atmosphere

(37 °C, 5 % CO_2). The absorbance of the formazan product generated was measured at a test wavelength of 450 nm using a multi-well plate reader. Compounds were dissolved in DMSO and the final solvent concentrations never exceeded 0.5 % (v/v). The control wells included treated cells with 0.5 % (v/v) DMSO and the positive control. 10 μM verapamil was included as a control for the HT-29 cell line. Measurements were performed in triplicate, and each experiment was repeated three times.

4.2.3. Cell cycle analysis

MCF7, HeLa, and HEK-293 cell cycle analyses were performed by quantifying the DNA content by flow cytometry. Cells in exponential growth phase at $10{\cdot}10^4$ cells/mL for MCF7, $5{\cdot}10^4$ cells/mL for HeLa and $4{\cdot}10^4$ cells/mL for HEK-293, were seeded in 6-well plates (2 mL/well). After 24 h incubation, cells were treated with compounds **8** at 1 μM and **60*Z*** and **67** at 100 nM. The effect of treated and untreated cells was measured 24, 48, and 72 h post-treatment. After the corresponding time, live and dead cells were collected and fixed in ice-cold ethanol/PBS (7:3) and stored at 4 °C for later use. Cells were rehydrated with phosphate-buffered saline (PBS), treated with 100 μg/mL RNase A (Sigma-Aldrich), and stained overnight in darkness at room temperature with 50 μg/mL propidium iodide (PI) (Sigma-Aldrich). Cell cycle profiles were then analyzed by flow cytometry using BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences). Data were analyzed with BD Accuri™ C6 Software (version 1.0.264.21) and compared to control cells. Compounds were dissolved in DMSO and the final solvent concentrations never exceeded 0.5 % (v/v). Control wells included cells with 0.5 % (v/v) DMSO.

4.2.4. Apoptotic cell death quantification

MCF7, HeLa, and HEK-293 apoptotic cells were quantified using an Annexin V-FITC/PI apoptosis detection kit (Immunostep, Salamanca, Spain) according to the manufacturer's guidelines. 1 mL/well of cells in the exponential growth phase at $5{\cdot}10^4$ cells/mL for MCF7, $2.5{\cdot}10^4$ cells/mL for HeLa, and $2{\cdot}10^4$ cells/mL for HEK-293 were seeded onto 12-well plates and left to attach overnight. After 24 h incubation, cells were treated with compounds **8** at 1 μM and **60*Z*** and **67** at 100 nM. After 72 h post-treatment, attached and floating cells of treated and untreated wells were collected, centrifugated, resuspended in the Annexin V binding buffer, and stained with Annexin V-FITC/PI. Cells were then incubated in darkness at room temperature for 15 min and a total of 5,000 cells of the selected population were acquired and analyzed using the BD Accuri™ C6 Plus Flow Cytometer and Software (BD Biosciences), respectively. Compounds were dissolved in DMSO and the final solvent concentrations never exceeded 0.5 % (v/v). Control wells included cells with 0.5 % (v/v) DMSO.

4.2.5. Confocal microscopy

MCF7 and HeLa cells were grown on square glass coverslips coated with poly-L-lysine (22 mm^2). To reach appropriate cell confluence, coverslips were manipulated in 6-well plates (1 coverslip/well) seeding 2 mL/well of cells in the exponential growth phase at $10{\cdot}10^4$ cells/mL for MCF7 and $5{\cdot}10^4$ cells/mL for HeLa cells. After 24 h incubation, cells were treated with compounds **8** at 1 μM and **60*Z*** and **67** at 100 nM and incubated for 24 h. Cells were washed with PBS, fixed with 4 % formaldehyde in PBS for 10 min, permeabilized with 0.5 % Triton X-100 (Boehringer Mannheim) in PBS for 10 min, and blocked with 10 % BSA in PBS for 30 min. Then, coverslips were incubated with anti-α-tubulin mouse monoclonal antibody (diluted 1:200 in 3 % BSA/PBS) (Sigma-Aldrich) for 1 h. After PBS washing, coverslips were incubated with fluorescent secondary antibody Alexa Fluor 488 goat anti-mouse IgG (diluted 1:400 in 1 % BSA/PBS) (Molecular Probes, Invitrogen) for 1.5 h in darkness. After being washed with PBS, a drop of ProLong™ Gold Antifade Mountant with DAPI (ThermoFisher) was added to preserve fluorescence and stain cell nuclei. Cells were analyzed by confocal microscopy using a LEICA SP5 microscope DMI-6000 V model coupled to a LEICA LAS AF software computer.

4.2.6. Tubulin isolation and inhibition of tubulin polymerization

Microtubular protein was isolated from calves' brains according to the modified Shelanski method[37,38] by two cycles of temperature-dependent assembly/disassembly and stored at −80 °C. Before each use, protein concentration is determined by the Bradford method taking BSA as standard.[39] Tubulin polymerization was monitored at 450 nm using a Helios α spectrophotometer by measuring the increase in turbidity caused by a 4 °C to 37 °C shift, which allows the in vitro microtubular protein to depolymerize and polymerize respectively. The assay was performed in quartz cuvettes containing 1.5 mg/mL microtubular protein and the ligand (except for the control cuvette with only DMSO at the same concentration) in a mixture of 0.1 M MES buffer, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM β-ME, and 1.5 mM GTP at pH 6.7 (final volume 500 μL). Cuvettes were preincubated at 20 °C for 30 min, to allow ligand binding to tubulin, and subsequently cooled on ice for 10 min. Then, the experiment starts at 4 °C to establish the initial baseline. The assembly process was initiated by a temperature shift to 37 °C and the turbidity produced by tubulin polymerization can be measured by an absorbance increase. After reaching a stable plateau, the temperature was switched back to 4 °C to return to the initial absorption values (to confirm the reversible nature of the monitored process and to determine whether or not the microtubular tubular protein has stabilized). The difference in amplitude between the stable plateau and the initial baseline of the curves was taken as the degree of tubulin polymerization for each experiment. Comparison with control curves under identical conditions but without ligands yielded TPI as a percentage value. All compounds were tested at 10 μM concentration. IC_{50} was calculated for compounds that inhibit tubulin polymerization by>50 % at 10 μM. Compounds were dissolved in DMSO and the final solvent concentrations never exceeded 4 % (v/v), which has been reported not to interfere with the assembly process. All the measurements were carried out in at least two independent experiments using microtubular protein from different preparations.

4.2.7. Western blot

Following treatment, around $4\text{-}5{\cdot}10^6$ cells were harvested and brought together. Cells were washed twice with PBS at 4 °C and centrifuged for 7 min at 1200 rpm between washes. The resulting pellet was resuspended in 60–120 μL of WCE-300 lysis buffer, supplemented with phosphatase and protease inhibitors (ThermoFisher Scientific). After a 20-min incubation in ice, the samples were centrifuged at 14000 rpm for 18 min at 4 °C to provide the whole cell protein extract (supernatant). Protein concentration was measured by Bradford procedure at 595 nm first establishing a calibration curve with BSA. Protein lysates (80 μg) were denatured by heating at 96 °C for 5 min in SPLB. Proteins were subsequently separated by molecular weight in a polyacrylamide gel (12 %) in SDS-PAGE under reducing conditions. A colorful protein ladder was run in parallel. After electrophoresis, proteins were transferred overnight at 4 °C to hydrophobic nitrocellulose membranes (Merck Millipore), previously activated in methanol for 5 min. Then, membranes were blocked with 5 % BSA or 5 % non-fat skimmed milk powder in TBS-T for at least 1 h at RT. The incubation with primary antibodies anti-phospho-Ser/Thr-Pro MPM-2 Antibody, clone MPM-2, Upstate®, from mouse (Merck Life Sciences) was conducted at 4 °C for around 16 h. After three 5-min TBS-T washes, membranes were incubated with anti-mouse IgG (whole molecule)–Peroxidase antibody produced in rabbit (Merck Life Sciences) for 1 h at RT. Then, protein bands were visualized in a ChemiDocTM Imaging System (BioRad) using the enhanced chemoluminescence detection kit SuperSignal™ West Femto (Thermo Fisher). Experiments were performed at least three times with independent protein lysates.

4.3. Computational studies

4.3.1. Ligand structure calculations

Calculations were performed using the Spartan 08 software package. The conformational searches were performed at the molecular mechanics (MMFF94s) level and the obtained conformations were energy minimized until convergence at the B3LYP/6–31 + G* DFT level.

4.3.2. Docking calculations

Docking studies were carried out as previously described.[26,40] Briefly, dockings were performed in parallel with PLANTS with default settings[34] and 10 runs per ligand and with AutoDock 4.2[35] applying the Lamarckian genetic algorithm (LGA) 100–300 times for a maximum of 2.5×10^6 energy evaluations, 150 individuals, and 27,000 generations maximum. RMSD between the retrieved poses and model scaffolds with no substituents and colchicine binding site ligands in complex with tubulin were calculated with LigRMSD.[41] The retrieved docked poses were automatically assigned to the colchicine subzones using in-house KNIME pipelines.[42] The programs' docking scores were converted into Z-scores and the poses with the best consensus scores were selected as the docking results. Docked poses were analyzed with Chimera,[43] Marvin,[44] OpenEye,[45] and JADOPPT [46].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2022.106282.

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